QUANTITATIVE ESTIMATION OF ZINC, COPPER, ALUMINIUM AND MANGANESE : HISTOLOGICAL AND HISTOCHEMICAL LOCALIZATION OF ZINC IN THE RAT BRAIN

DISSERTATION SUBMITTED FOR THE DEGREE OF

Master of Philosophy

IN

ANATOMY

BY

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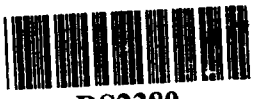

Dedicated
to my
Parents

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This is to certify that the research work embodied in the Dissertation, entitled "Quantitative Estimation of Zinc, Copper, Aluminium and Manganese : Histological and Histochemical localization of zinc. In the Rate Brain", has been carried out by Mr Mohammad Hossein Lotfian under my guidance at Brain Research Center, Jawaharlal Nehru Medical College, Aligarh. In my considered opinion this work is suitable for submission for the award of the degree of Master of Philosophy (M.Phil) in Anatomy of the Faculty of Medicine of Aligarh Muslim University, Aligarh.

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CONTENTS

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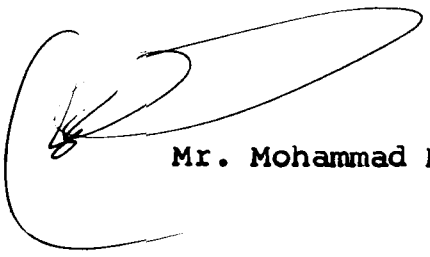

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ABSTRACT

This study was undertaken to understand complex chemical distribution of cellular zinc in the rat brain.

In quantitative analysis the regional distribution of zinc levels in the rat Brain were determined by the help of atomic absorption spectroscopy.

Histological and histochemical attempts were made to bring to the surface the latest problems in the histochemical techniques for demonstration of zinc in the rat brain.

There is little doubt that the field of zinc neurobiology owes a part of its existance to striking image of zinc-labelled mossy fibres coursing through the hippocampus, which are richly laden with large amounts of the zinc.

Light microscopical studies has been focussed on histological and histochemical distribution of zinc in the hippocampus and the mossy fibres in the rat brain.

Introduction

1- INTRODUCTION

What is termed as trace element includes certain metals, like Zinc, Copper, Aluminium, Manganese and Cobalt which are found in very minute quantity in the environment. But this term is no longer strictly tenable in the industrialized countries like Japan, where dumping of man-made poison, or in other words industrial waste product, is one of their major problem. These waste products eventually find their way into the rivers and cause poisoning of natural environment.

The trace element of the past is no longer in trace quantity in our present natural environment. Environmental protection in the form of safe disposal of industrial waste material is one of the most important task of our present generation. If we change the balance of the nature by introducing these elements into the nature in the form of industrial waste product, it will not be very far to see the fruitful plants of our present time will turn to poisonous plants for our future generation.

Reports of zinc concentration after parental administration of single dose of labelled zinc (Sheline et al 1943, Hasan, 1976) indicate that brain accumulates substantial amounts of the metal.

To the neurobiologist, recognition of the constant occurence of several trace metals in the brain signalled the need to examine their possible structural or functional significance in neural tissues. Of 15 trace elements currently deemed to be essential, zinc has received most attention in recent times because of the association of the metal with some 50-100 enzymes (Chelebowski and Coleman 1976).

Hurley and Swenerton (1966) have linked 'in utero' zinc deficiency in rats to abnormal fetal development, especially of the central nervous system. Zinc is required during neurogenesis and for normal formation of central nervous system. The teratogenicity of zinc deficiency is widely ascribed to impaired nucleic acid synthesis during embryonic development (Swenerton et al 1969).

The total zinc content of normal 70 kg man is approximately 1.4 to 2.3 g (Widdowson et al, 1951). The metal is present throughout all body fluids of tissues (Tiptons, Cook, 1963) and is homogeneously distributed in most organs which contain about 20 to 30 µg zinc/g wet weight of the tissue.

Zinc was first shown to be an essential nutrient for mammals by Todd et al, (1934). Interest in zinc metabolism in man focused largely on its toxic properties. It was considered that ubiquity of zinc in the environment made human deficiency unlikely. However, in 1958 Prasad suggested that the syndrome of dwarfism and hypogonadism, seen in adolescent males in Iran, might be due to a nutritional

deficiency of zinc (Prasad et al., 1961). Since then there have been many reports of zinc deficiency occurring in groups of people in widely differing circumstances and in many different countries.

1.1 Zinc as an 'Essential Nutrient'.

1.1.1 Body Content and Metabolism:

The highest concentrations (100 to 200 ppm) occur in the eye, hair, bone, and male reproductive organs, with intermediate concentrations (40 to 50 ppm) in the liver, kidney and muscle (Underwood, 1977). In the blood 80 % of the total zinc is in the red cells. The main plasma zinc level is about 90 µg/100 ml, serum zinc being about 10 % higher. About one half of the plasma zinc is in a freely exchangeable form, loosely bound to albumin. Most of remainder is tightly bound to α_2 macroglobulim, with 70 % of the total bound to amino acids, the most important of which are histidine and cysteine (Beisel et al 1972).

Absorption of zinc takes place mainly in the duodenum. The mechanism appears to be an active transport involving a small binding lingand of pancreatic origin. This ligand facilitates the uptake of zinc from the lumen of the small intestine into the epithelial cells. The element is then transferred to a binding site on the basolateral membrane, which functions as a zinc donor to plasma albumin (Evans,

1976). The percentage of zinc absorbed from the diet has been reported to vary from 20 to 80 %, most values being between 20 to 30 % . Zinc absorption is dependent partially on the level of intake and on zinc nutritional status, a high percentage being absorbed in a deficient state.

The principal route of excretion is via the intestine. Fecal zinc consists of unabsorbed zinc with a small amount of endogenous origin, mainly from pancreatic exocrine secretions. Urinary zinc losses are small, amounting to 0.3 to 0.5 mg/24 hr in the normal, healthy adult (Hambidge & Walravens, 1975). Sweat losses are normally low but may become important in hot climate or situations that produces copious sweating, under such conditions losses of 4 mg Zn/day may occur (Prasad et al.1963a).

1.1.2 Biochemical and Physiological Role:

More than 20 different zinc metalloenzymes have been identified, including carbonic anhydrase, alkaline phosphatase, and alcohol dehydrogenase. Many other enzymes are known to be activated by zinc (Parisi & Vallee 1969). Zinc has an important role in the metabolism of proteins and nucleic acids. It is apparently essential for the synthesis of DNA and ribosomal RNA; RNA polymerase is a zinc metalloenzyme (Fernandez Madrid et al., 1973). Zinc also has a role in mitotic cell division and in the ultilization of amino acids in protein synthesis, particularly of collagen in skin and

scar tissue (Mc Clain et al., 1973). Although little is known of the relation between the biochemical role of zinc and the clinical features of zinc deficiency, some of these features may arise from disturbances of nucleic acid and protein metabolism.

Numerous experimental studies with rats have shown an absolute requirement for zinc for growth, and its deficiency has severe effects on all stages of reproduction and of tissue proliferation in the young (Hurkey & Schrader 1972). Reproductive performance in both males and females is impaired by zinc deficiency. The effect on the young depends on the duration and timing of the zinc deprivation. In early gestation, zinc deficiency may cause severe congenital abnormalities. Later in gestation,it can cause growth retardation and impairment of brain growth, leading to impaired behavioral development after birth (Mc Kenzie et al, 1975). Feeding a low zinc diet to lactating dams produces symptoms of zinc deficiency including impaired growth, in suckling pups (Mutch & Huley 1974). Zinc deficiency is not yet recognized as a cause of human congenital malformation but this possibility has been suggested on the basis of epidemiology. Impaired sexual maturation in adolescent males and poor growth in young children have been observed in human zinc deficiency. Zinc has also been shown to have a role in the growth of hair, skin, and bone.

1.1.3 Availability:

Zinc is less available for absorption from plant foods than from animal foods, one of the factors responsible may be the high phytate (inositol hexaphosphate) phytic acid content of many plant foods, particularly grains. Phytate binds strongly to zinc, markedly decreasing its availability (Halsted et al 1974). Recent studies indicate that fibre, particularly in unleavened breads, binds strongly with zinc, making it unavailable for absorption (Reinhold et al 1976). Other plant constituents, such as hemicelluloses and amino acid-carbohydrate complexes, may also be involved.

1.1.4 Requirements:

The precise minimal requirements for zinc for optimal health and growth are not known. These depends, in part, on the composition of diet. The source of protein may affect the zinc requirement as much as four-fold, the variation arising from the lower availability of zinc in plant foods. Various physiological conditions for example, pregnancy, lactation, and periods of rapid growth and development in infants, children, and adolescents - are associated with a relatively high zinc requirement.

1.2 Zinc Deficiency in Man:

1.2.1 Aetiology:

Nutritional deficiency, due to marginal zinc intake may be quite common even in countries such as the United States (Sandstead, 1973). In rural areas of Egypt and Iran, where human zinc deficiency was first identified, dietary intakes of zinc appeared initially to be quite adequate (Sarram et al, 1969). In these rural areas unleavened (flat) bread, madewithout yeast to raise the dough, is a principal part of the diet. In larger towns where yeast leaving is used, the incidence of zinc deficiency is much lower suggesting an explanation for this observation. This highly localized nutritional deficiency is ascribed to the consumption of a diet very high in cereal grains, cereals contain large amount of phytic acid (myoinositol hexaphosphate) a substance that binds divalent metal cations especially Zn^{2+} very tightly. Thus, decreased availability may contribute to the development of a nutritional zinc deficiency.

High urinary losses of zinc over an extended period may lead to zinc depletion. Urinary excretion of zinc appears to vary directly with the amount of the element bound to the plasma amino acid pool. Conditions that increase the circulating amino acids, particularly histidine and cysteine, also cause an increase in renal clearance of zinc (Morrison et al 1978). Increased urinary excretion has been reported

in a number of condition, including alcoholism and post alcoholic cirrhosis (Sullivan & Lankford, 1962) and other liver diseases. Hyperzincuria occurs in any condition associated with increased catabolism, such as surgery, burns, multiple injuries, major fractures, diabetes mellitus, protein deprivation, and starvation (Spencer et al, 1976). Excessive urinary excretion also results from abnormal excretion of molecules to which zinc binds and which prevent it from being reabsorbed, for example, albumin in nephrotic syndrome and chelating agents such as ethylene-diamine-tetra-acetate (EDTA) and penicillamine (Sands leads et al, 1976).

Poor zinc status may be associated with several genetic diseases including thalassemia and sickle - cell anemia (Prasad et al, 1975a) but only one inherited disorder of zinc metabolism causing a deficiency syndrome has been described. This is acrodermatitis enteropathica (AE) an autosomal recessively inherited disease, the symptoms of which are consistent with severe zinc deficiency (Neldner and Hambidge 1975).

1.3 Clinical Manifestation:

The signs and symptoms of zinc deficiency depend, to some extent, on age, acuteness of onset, duration and severity of zinc depletion, and the circumstances in which the zinc deficiency occurs. Many of the features observed in

man are similar to those seen in zinc-deficient animals. Chronic deficiency in Pediatric and adolescent age groups causes retarded growth. In adolescence, sexual maturation is delayed. Anorexia, pica, impaired taste acuity, and mental lethargy have all been reported to occur. Other disturbances may include rough, dry skin, impaired wound healing, and increased susceptibility to infection. Abnormal glucose tolerance has been observed in the Middle East (Sandstead et al, 1967), and impaired secretion of luteinizing hormone has been reported. Acute zinc deficiency can occur in patients on long-term total parental nutrition (TPN), on penicillamine therapy, and in acrodermatitis enteropathica (AE).

1.4 Diagnosis and Treatment:

Zinc deficiency may occur in conditions as a result of altered zinc metabolism, rather than zinc deficiency such condition include infections and hypoproteinemia, as well as the use of oral contraceptive (Beisel and Pekarek, 1972). Similarly, there is no evidence to indicate that the hypozincuria observed in Down's syndrome, pernicious anemia, and various cancers is associated with a deficiency state.

Hair zinc concentration reflects past dietary intake, but may be complicated by a decline in the rate of hair

growth. Measurements of zinc concentration in other tissues and fluid may also be helpful in detecting or confirming zinc deficiency. These include erythrocyte and parotid saliva zinc level and urinary excretion. Demonstration of reduced activity of a zinc dependent enzyme would also provide a biological index of zinc depletion. Unfortunately, the zinc metalloenzymes most sensitive to deficiency include pancreatic carboxypeptidase and liver alcohol dehydrogenase, which are not readily accessible. Serum alkaline phosphatase may be useful, however, as activity is frequently decreased in zinc deficient states and increases after supplementation.

In most circumstances oral administration of 1 mg Zn^{2+}/Kg. body weight per day is adequate to treat zinc deficiency (Hambidge and Walravens, 1975). This amount is within the range of normal daily intake of zinc, and no side-effects have been reported. Zinc is equally well-absorbed as the sulfate or acetate, although the former may cause gastrointestinal irritation, which is reduced if the sulfate is taken with meals. Both forms are soluble and thus can be administered in a liquid, which is more suitable for children. Intravenous requirements for patients maintained on total parental nutrition (TPN) appear to be about 28 to 30 mg Zn^{+2}/Kg per day. In acute zinc deficiency syndromes, improvement in the patient's condition may occur very rapidly with the institution of zinc therapy. Mood changes have been

observed within a few hours, diarrhoeamay be completely controlled within 3 to 4 days, and skin lesions may clear up in 2 weeks (Kay and Tasman Jones, 1976).

1.5 Epidemiological Aspects of Zinc Deficiency

1.5.1 Fetus:

Maternal zinc deficiency has also been suggested as a factor in the high incidence of anencephaly in Alexandria, Egypt, and Shiraz, Iran (Sever, 1973). A high incidence of congenital malformations has been observed in adult women suffering from AE (Hambidge, et al, 1975).

1.5.2 Infants:

The new-born infant does not appear to have a store of zinc like the reserves of iron and copper, and tissue concentrations are similar to adult levels (Casey & Robinson 1978). Large negative zinc balance arising partly from high urinary losses, have been reported in the first weeks after birth (Cavell & Widdowson, 1964) plasma zinc levels in young infants have been found to be dependent on dietary intakes, and indicate suboptimal zinc nutrition.

1.5.3 Preschool Children:

Pre-school children (1 to 4 years).

A study in Denver found that mean hair zinc levels of

pre-school children from middle-income families were lower than those of older children and adults (Hambidge et al, 1972). In low income families, poor zinc nutrition may be one of the environmental factors responsible for the high incidence of low growth incidenceseen in this population group.

1.5.4 Adolescents:

Zinc deficiency was first recognized in 1961 as being a major factor in the syndrome of nutritional dwarfism seen in adolescents in rural areas of Iran and Egypt (Prasad et al, 1961). About 3 % of the adolescent population in these areas may be affected and a similar syndrome is found in other countries, including turkey, Tunisia, Moracco, Portugal and Panama (Halsted et al, 1974). One factor contributing to the zinc deficiency in the Middle East is the widespread use of unleavened breads as a major staple food. These breads are made from flours of very high extraction rate and contain considerable amount of phytate and fibre.

1.5.5 Acrodermatitis enteropathica (AE)

Acrodermatitis enteropathica (AE) is a familial disease with an autosomal recessive inheritance. The disorder is characterized by skin lesions, diarrhoeaand alopecia, symptoms consistent with sever zinc deficiency. The disorder is effectively treated with oral zinc therapy (Neldner and

Hambidge 1975). The basic molecular defect has not yet been identified but appears to be a in the intestine where it causes a partial block in zinc absorption (Lomback et al, 1975a).

1.6 Cause of Zinc Deficiency:

1.6.1 Nutritional:

In 1958, a 21 year-old patient at Saadi Hospital Shiraz, Iran, was brought to the attention of Prasad et al. (1961). He looked like a 10 years old boy. Besides dwarfism and hypogonadism he had hepatosplenomegaly, rough and dry skin, mental lethargy, and geophagia (Prasad et al, 1961). He ate only bread made of wheat flour, and his intake of animal protein was negligible. Geophagia (clay eating) is common in the villages in Iran. During a short period of time, Prasad et al (1961) found 10 similar cases.

Although there was no evidence of blood loss, the patient was anemic, and it was determined after extensive investigation that the anemia was related to a nutritional deficiency of iron. It was concluded that the iron deficiency was due to a lack of iron availability from the bread, greater loss of iron caused by excessive sweating in the hot climate, and the adverse effect of geophagia on iron absorption. In every case the anemia was completely corrected by the administration of oral iron but growth retardation and testicular atrophy are not seen in iron deficient

experimental animals, the possibility that zinc deficiency may have been a complicating factor was considered. Since heavy metals may form insoluble complexes with phosphates, it was considered possible that these heavy metals may form insoluble complexes with phosphates. Furthermore, it was possible that the factors responsible for decreased iron availability in these patients may also adversely affect zinc availability.

Further studies in Egypt and Iran showed that the growth rate was greater in patients who received zinc than in those who received iron or only animal protein diet (Sandstead, and Prasad, 1967). Genitalia size became normal, pubic hair appeared, and other secondary sexual characteristic develped within 12 to 24 weeks in all subjects receiving zinc. No such changes were observed in a comparable length of time in the iron supplemented group or in the group on an animal protein diet alone. Thus, the growth retardation and gonadal hypofunction in these subjects were related to zinc deficiency.

1.7 Zinc and Brain Development:

Zinc deficiency in pregnant rats was shown to cause fetal abnormalities, behavioural impairment in the offspring, and maternal difficulty in parturition (Hurley and Schradar 1972). It was proposed that impaired deoxyribo-nucleic acid (DNA)

synthesis in zinc deprived embryoes prolongs the mitotic cycle and reduces the number of normal neural cells, leading to malformations of central nervous system. It is tempting to speculate that the exceptionally high rates of congenital malformations of the central nervous system reported from the middle East (Damyanoy and Duter 1971) might be caused by maternal zinc deficiency Caldwell et al (1970) were the first to show that in both prenatal and postnatal nutrition even mild zinc deficiency in rats had a profound influence on behaviour potential, despite an apparently adequate protein level in the diet.

Palsson and Grisson (1953) found that the offspring of ewes fed on sea-weed showed symptoms and morphological characteristics of enzootic ataxia. The lamb had demyelination of the cerebrum and other abnormalities of the brain Although the dietary copper intake was adquate the pregnant ewes showed low level of the element in blood and in affected lambs the liver copper content was subnormal.

Guinea pig Abnormalities of the nervous system, similar to those seen in lamb with enzootic ataxia, were found in the new born off-springs of female guinea pigs fed a copper deficient diet during growth and pregnancy. Those offsprings which had subnormal level of liver copper, showed a high incidence of ataxia and gross abnormalities of the brain at birth. The brain of copper deficient pups were pale and translucent, with many small haemorrhages areas.

Cerebellar folia were often absent or abnormal in appearence. Throughout the brain there was a paucity of myelin, which was abnormally low in phospholipid concentration. The pups that survived birth usually died within the first month of life of aneurysms of the aortic arch.

The newborn pups from copper deficient dams were found to be hyperirritable, with convulsive seizeures following stimulation. Gross neural lesions included focal pale areas in cerebral cortex and corpus striatum, prominent cerebral odema, and cortical necrosis. Unlike lambs afflicted with enzootic ataxia, the rats did not show nerve fibre degeneration in the brain-stem or spinal cord (Carlton and Kelly 1969).

It is known that small amount of metabolic copper from intrauterine loops or wires can prevent mammalian embryogeries is by blocking implantation and blastocyst development (Change et al, 1970).

Menke's kinky hair syndrome in human beings is an X-linked genetic disorder, was first described in 1962 (Menkes 1962). The disease is characterized by progressive degeneration of the brain and spinal cord in infants.

1.8 Toxicity:

Three types of toxic reactions to zinc have been reported in human beings. First "metal fume fever characterized by pulmonary manifestation, has been reported to occur

in industrial workers exposed to fumes the second type of toxicity was observed in a 16-year-old Iranian boy who ingested 12 g zinc sulfate over a period of 2 days. The third type of acute zinc toxicity involved a patient with renal failure after hemodailysis (The water for hemodialysis was stored in galvanized tank). The patient suffered from nausea, vomiting fever and severe anemia. Vomiting, a protective phenomenon, occurs after ingestion of large quantities of zinc in fact 2 g zinc sulfate has been recommended as an emotic.

The symptoms of human zinc toxicity include dehydration electrolyte imbalance, abdominal pain, incoordination. Acute renal failure, caused by zinc chloride poisoning, has been reported. The symptoms occurred within hours of ingesting large quantities of zinc. Death is reported to have occurred after ingestion of 45 g of zinc sulfate.

1.9 Different forms of zinc in the plasma:

Almost all of plasma zinc is loosely or tightly bound to protein molecules (Parisi and Vallee 1970) reported that the primary zinc - binding protein of the plasma is an 2-α macroglobulin that accounts for about 40 % of the total zinc. Smaller percentage may be tightly bound to enzymes or proteins such as transferring and about 7% is bound to plasma amino acid, primarily histidine and cysteine (Prasad and Oberleas 1970).

The remaining plasma zinc exists as a loosely bound albumin complex.

1.10 Metabolism and Bio-chemistry:

Zinc in Plasma is mostly present as bound to albumin, but other proteins, such as α_2 - macroglobulin, transferrin ceruloplasmin haptoglobin and gamma globulins, also bind a significant amount of zinc (Prasad and Oberleas 1970). Besides this protein-bound fraction, a small proportion of zinc (2 to 3 % of oral Zn) in the plasma exists as an ultrafilterable fraction bound to amino acids but with a smaller amount in ionic form. Over 70 metalloenzymes are known to require zinc for their function (Riordan 1976). The metal is present in several dehydrogenoses, of aldolases, peptidases and phosphatases.

Zinc deficiency in animal impairs the incorporation of labeled thymidine into DNA.

RAN polymerase is a zinc-dependent enzyme (Terhune and Sandstead 1972). The fact that the activity of RNase is increased in zinc - deficient tissue (Prasad and Oberleas 1973) suggests that the catabolism of RNA may be regulated by zinc.

Activity of alkaline phosphatase was found to be reduced in bones from zinc-deficient rats. Pigs, Chicks, Turkey Poults (Kirchgessner et al, 1976). Two important zinc metalloenzymes in protein digestion are pancreatic carboxy-peptidase A & B.

Zinc deficiency lowers the activity of alcohol dehydrogenase in liver, bones, testes, kidney, and oesophagus of rats and pigs. (Prasad and Obarleas, 1971).

Zinc is required for the metabolism of vitamin A as well as the catabolism of ethanol.

Zinc may form mercaptides with third group of proteins, possibly linking to the phosphate moiety of phospholipids or interacting with carboxyl groups of sialic acid or proteins on plasma membrane, resulting in changes in the fluidity and stabilization of membranes.

The role of Ca^{++} in the function of the cell microskeleton, represented by microtubules and microfilaments, has been well documented. The contractile elements of this system are responsible in some way for the mobility of microorganelles and transport of granules to the membranes as well as the excitability of plasma membrane it-self. Zinc may compete with calcium and there-by inhibit the calcium effect. Zinc may intervene in non-enzymic free radical reaction (Editorial 1978) in particular, zinc is known to protect against iron-catalyzed free radical damage.

Divalent transitional - metal ions, such as Co^{++} Cu^{++} Fe^{++} or Fe^{+++} Mn^{++} may play an important role in the regulation of neural excitability.

In hippocampus, synaptic vesicles contain a high content of Zn^{++}. Zinc ions may potentiate the action of excitatory neurotransmitters (Smart & Constanti, 1983) by binding

to the sulfhydral group of GABA receptors (Smart Constanti 1982). Secondly, they may also interfere with a rapid uptake of released transmitters so causing an accumulation of these amino acids in the extracellular space. A sudden increase in the extracellular Zn^{++} concentration may profoundly shift the pH of the microenvironment, so exerting toxic influence on nerve cells. In this context, we note that the hippocampal CA_3 neurons, whose afferents contain high concentration of Zn^{++} (Frederickson et al 1983), are among the first group of cells damaged by neuro-toxins such as kainate (Nadler 1979).

Zinc has been found in high concentration in hippocampus and in the cerebellum (Hassler and Sorenmark 1968). The anatomical localization responsible for the zinc-induced lethargy is unclear but a diffuse encephalopathy is a possibility (Murphy 1970).

Hasan, (1976) has reported significant increase in the concentration of zinc in cerebrum, medulla, cerebellum and hippocampus after intreperitoneal administration of 5 mg/kg elementals zinc (in the form of $ZnCl_2$) for 3 to 7 consecutive days to albino rats.

Murphy (1970) has described the interesting case history of a 16 year old Iranian boy who had ingested 12 g elemental zinc. He suffered from unusual lethargy, lightheadedness, slight staggering of gait and difficulty in writing legibly although the exact anatomic localization

of zinc induced lethargy was unclear. Murphy (1970) postulated a diffuse encephalopathy as a possibility.

Observations of Hasan (1976) suggest that zinc occurs in higher concentration in medulla oblongata than in hippocampus, after zinc intoxication in male albino rat. He offered an explanation for the drowsiness and clouding of consciousness reported by Murphy (1970) in the case of accidental zinc intoxication, the brain-stem reticular formation could well be depressed by accumulation of zinc in the medulla oblongata.

Thallium, nickel and cobalt have been shown to increase the rate of lipid peroxidation thereby causing significant accumulation of lipofuscin (Hasan and Ali 1981). Lipid peroxidation in vivo has been claimed to be of basic importance in aging, in damage to cell by air pollution, heavy metals and organophosphate pesticide intoxication, (Hasan & Ali 1981).

Transmission electron microscopy studies suggest that Thallium-induced lipofuscin formation in hippocampus, irregular electron-dense bodies and coated vesicles were observed in these neurons (Hasan, et al., 1977). In hypothalamus, the neurons showed increased incidence of pigment granules and arcuate conformation of Golfi cisternae and vesicles (Hasan et al, 1977a).

The neurons of mamillary body were found to be studied with pleiomorphic lipofuscin granules, (Hasan and Glees, 1974), In area postrema there was increased occurrence of oliodendrogliacytes with vacuolated electron dense bodies.

with small round profile resembling sequentrated part of a nucleus was also seen.

1.11 Zinc dependent enzymes:

1.11.1 Effect of Zinc-deficient diet on enzymes

Thymidine kinase, alkaline phosphatase, and carboxy-peptidase are affected adversely within 6 days of instituting a zinc-deficient diet in experimental animals. Diminished activity of thymidine kinase may be an important factor responsible for reduced mitotic activity (Fujioka, Lieberman, Prasad et al 1974). Since the enzyme is widely recognized to represent a rate limiting step in DNA biosynthesis.

1.11.2 Insulin

The administration of insulin in rats brought about a significant rise in the zinc contents of the cerebellum pons pancreas and other organs (Ribas et al 1978).

1.11.3 Growth hormone (GH)

Growth hormone (GH) level in plasma is inversly proportional to the renal zinc excretion (Prasad et al 1969;Henkin,1974b).

1.11.4 Alkaline Phosphatase:

Roth and Kirchgessner (1974a) found the activity of alkaline phosphatase to be decreased by as much as 25 % after just 2 days of dietary zinc depletion and by 50 % after 4 days. This loss of activity was not due to reduced food intake; in fact the activity of this serum enzyme had already diminished before growth and food intake noticeably decreased.

In the peripheral and central nervous systems, barrier tissue exists, that limits the exchange of protein, ions and water soluble non-electrolytes between the parenchymal and vascular compartments (Ollsson and Reese' 71 Bradbury, 79; Weerasuriya et al 80) Tight junctions (zonulae occludentes) between cells in these tissues prevent paracellular diffusion of substances between compartments (Akert et al, 1976; Brightman, 1977). The barrier system of the peripheral nerve, the blood-nerve barrier (BNB) is formed in the endothelial cells of endoneurial blood vessels (Ollsson 71. Michel et al, 84a,b) and by the perineurial cells of the perineurium (Akert et al, 76), whereas blood-brain barrier (BBB) is located at cerebral blood vessels (Brightman, 77), the choroidal epithelium (Van Deurs and Koehler, 79) and arachnoid membrane (Van Rybrock and Low, 82).

Carole H. et al, (1987) have shown that small cerebral blood vessels in the rat had Alkaline Phosphatase reaction product associated with the endothelial cell membranes. In

the meninges no reaction product was detected in the dura, pia, or arachnoid trabecular. However, in the outer arachnoid membrane, the reaction product filled the extracellular space and caveolae.

Alkaline phosphatase was always present in at least one of the barrier tissues that separate the parenchyma of the peripheral and central nervous system from neurones of the peripheral and central nervous systems from non-neuronal components. In the central nervous system, AP was detected on both the luminal and abluminal surface of the blood vessels, and in the outer arachnoid membrane of the rat. The distribution of these enzymes in cerebral endothelial cells suggests that they are related to transport and regulate barrier function (Betz et al, '80, Mrsulja and Djuricic '79).

1.11.5 Carbonic anhydrase:

In the rat, activity of carbonic anhydrase was reduced by about 20 to 40 % within 2 and 4 days respectively, after feeding them a zinc-deficient diet (Roth and Kirchgessor 1974).

Carbonic anhydrase was first reported in brain tissue by (Van Goor 48). Histochemical studies have revealed carbonic anhydrase activity in glial cells (Giacohini, 61), chroid plexus (Korhonen et al 64 Maren and Vogh 80) and Muller cells of retina (Linser and Moscona 81). Carbonic anhydrase regulates the ion exchanges and acid-base balance

in the brain.During cerebrospinal fluid (CSF) formation, carbonic anhydrase in the choroid plexus provides H^+ to exchanges with Na^+ from blood into the CSF and it is apparently for this reason that inhibition of carbonic anhydrase reduces CSF pressure (Tschirgi et al,'54, Fishman,'81).

Vivien Wong, et al (1987) has reported that in the spinal cord carbonic anhydrase reactivity was found in glial cells throughout the grey and white matter as well as in axis cylinders of dorsal horn. In the brain medulla oblongata glia were highly reactive especially in the nucleus solitarius and locus ceruleus where their processes were especially distincted outlined.In the mid-brain, carbonic anhydrase reactivity was absent, in all neurons in the cerebellum intense glial reactivity was unaccompanied by neural staining. The choroid plexus and ependyma of the lateral ventricles showed carbonic anhydrase reactivity while the ependymal cells of the central canal of spinal cord were non-reactive.

Huber and Gershoff (1973) have reported depressed carbonic anhydrase activity and increased alkaline phosphatase in the tissue of rat fed high zinc diets.

1.11.6 Carboxypeptidase A and B:

Two additional zinc metalloenzymes important in protein digestion are the pancreatic carboxypeptidases A & B. In zinc deficiency studies caboxy-peptidase A showed a reduced activity in pancreas of rats (Prasad and Oberleas, 1971).

1.11.7 Dehydrogenases:

The lactate, malate, alcohol, and glutamat dehydrogenases are other zinc metalloenzymes that differ in their molar content of zinc. They show, depending on species and tissue, unchanged or slightly reduced activities in response to zinc deficiency (Kichgessor et al, 1976a).

1.11.8 RNA and DNA polymerase:

Zinc occurred in several highly purified preparation of RNA and DNA from very different sources.

The activity of the DNA-dependent RNA polymerase on the brain of prenatal zinc-deficient rats, reduced RNA Polymerase activity was also found, in addition to smaller brain size and diminished DNA synthesis (Sandstead et al, 1972).

1.11.9 Ribonuclease:

Testis of zinc deficient rats contained less zinc, RNA, DNA and protein, but at the same time exhibited an elevated ribonuclease activity (Somers and under wood 1969). The primary defect is also increased ribonuclease activity, which led to increased protein catabolis.

1.11.10 Thymidine kinase:

More recent studies have showed that zinc is also

required for the activity of thymidine kinase, an enzymes essential for DNA synthesis and hence also for cell division.

In zinc deficiency, the tissue concentrations of other essential trace elements, besides zinc, may also be greatly affected (Kirchgessner et al, 1978).

Diminished activity of zinc dependent enzyme thymidine kinase may be an important factor responsible for reduced mitotic activity (Prasad et al, 1974).

1.11.11 2'-3' Cyclic nucleotide phosphohydrolase:

Myelin maker enzyme 2'-3' cyclic nucleotids phosphohydrolase and the brain alkaline phosphatase have a role in processes of myelination and brain maturation (Cohen, 1970). In this connection the report of apparently improved myelination following zinc therapy in a 6 month-old human acrodermatitis enteropathica infant is especially note-worthy (Ohlsson 1981). Changes of glutamate dehydrogenase may be of consequence in relation to metabolism of neurotransmitter glutamic acid and to glutaminergic neural system.

1.11.12 Cuprein (2Cu, 2Zn, Superoxide dismutase);

In the biochemistry of oxygen, the reactivity of metal proteins dealing with reversible oxygenation, the metabolism

of peroxides and the formation of water are fairly well understood. Using human tissue, surprisingly, 2 additional g-atoms of zinc were found to be bound to the protein portion. Cuprein prevents the production of singlet oxygen in different systems (Weser and Paschan 1972), low molecular superoxide and hydrogen radicals and singlet oxygen. As Mc-Cord (1974) had postulated that hydroxyl radical products via the reaction of superoxide with hydrogen radical were responsible for the inflammatory type of arthritis.

The cuprozinc enzyme superoxide dismutase, which protects against damage by superoxide radicals, is also of interest as reference has been made to the sharply increasing activity of this enzyme and that of the mangano-form of superoxide dismutase in the first two months postnatally in rat brain (Mavelli et al 1982). Attention has been drawn to the possible developmental consequences that could arise if activity of this enzyme was reduced in the early neonate (Mavelli et al 1978). In addition, evidence has been presented of declining level of superoxide dismutase in aging rat brains and mention has been made of the attendant membrane injury which might accompany this event (Vanella et al, 1981). Of particular interest is the observation that activity of superoxide dismutase appears to be especially high in synaptosomes, since superoxide anions can cause oxidation of catecholamines. Superoxide dismutase

should, therefore, protect catecholamines. Dietary copper depletion has been demonstrated to reduce superoxide dismutase levels in rat brain (Morgan, O'Dell, 1977). The level of norepinephrine and dopamine were higher in the brains of zinc deficient weanling rats - an observation that led them to suggest that behavioral changes in zinc-deficient animals may in part be catecholamine related.

Coincidentally with the behavioral and neurochemical studies a particular interest developed with respect to the hippocampus, as certain of memory and learning defects associated with zinc deficiency resembled aspects of the behavioral syndrome resulting from ablation of the hippocampus. Furthermore, trace element analyses and histochemistry of the brain had consistently shown the hippocampus to be a region especially rich in zinc, which was found to accumulate in the intrahippocampal mossy fibre pathway during first 2-4 weeks postnatally (Crawford, Connor 1972).

1.12 Zinc and tubulin:

The involvement of zinc in the polymerization of tubulin into micro-tubules (Torre et al, 1981) and the induction by zinc of microfilament and microtubule protein has been shown (Eagle et al 1983). Both these structures are considered to play important role in the formation of the neural fold

(Dryden 1978). Microtubule reassembly was reduced in brain extracts from zinc deficient animals due to impaired ability of tubulin to repolymerise.

1.13 Protective action of zinc:

Among the first to note such effects were Willoughby et al. (1972). In young growing horses grazing on pastures contaminated with both lead and zinc from nearby refinery effluents, these authors found that the signs and symptoms of lead poisoning were considerably mitigated. Although the tissue lead contents were nearly doubled, the horses showed less evidence of intoxication than animals exposed to lead alone.

A similar protective effect of zinc against lead intoxication in young rats was noted by Cerklewski and Forbes (1976). As the dietary zinc content was increased, the severity of lead toxicity decreased. Furthermore, lead concentrations were lowered in the blood, liver, kidneys, and bones. Explanation offered was that the increased dietary intake of zinc impaired the intestinal absorption of lead thereby blocking its action. Such reasoning, however, would not explain the report of Willoughby (1972), in which enhanced absorption of lead, as well as lessened symptoms of lead toxicity, were noted in the zinc protected animals.

1.14 Transport of Zinc into the Brain:

On the basis of the available evidence, it appears that Zn enters the brain via transport sites which are as yet anatomically undefined, but nevertheless respond to lowered circulating levels of plasma Zn by increasing net transport. Zn is likely to be taken up by neurons (and undoubtedly glia as well) and a proportion of it transported distally by slow axonal transport (Knull, 1975).

According to Haskins, (1980), it is tempting to specu-late that Zn may be transported in association with tubulin, Transport of Zn is a general property of all neurons, then Zn would be projected widely along anatomic path-ways after entry into the CNS and thereby contribute to the relatively uniform distribution of Zn in brain.

In addition to a potential role in microtubular function, it is probable that Zn is incorporated into Zn metalloenzymes or other structural ligands either in the neuronal soma, the axon or synapse. Further study is clearly needed to define the function of Zn in brain on a molecular basis. Excess Zn appears to be eliminated via CSF pathways and probably reenters plasma by transport through the choroid plexus. However these experiments do not exclude the possibility that Zn egress may occur through other barrier tissue such as cerebral capillaries.

Peripherally administered zinc became bound rapidly to albumin, α-macroglobulin, transferrin, haptoglobulin, γ-globulins, probably other proteins (Prasad, 1979) and hence was unavailable to be transported to the CNS. Secondly, since the concentration of zinc in all areas of the brain, including the hippocampus, was high, it was postulated that under physiological conditions zinc in the brain does not exist in free form and must exist mostly in bound form. Intracerebroventricular administration of zinc sulfate or zinc chloride caused epileptic seizures characterized by running fits, jumping myocolonic movements of the limbs, and tonic-clonic convulsions. These symptoms were blocked or reversed by γ-aminobutyric acid. Activity of glutamic acid decarboxylase decreased significantly in hippocampus following 15 to 30 min., periods after the administration of zinc, it was due to reduced concentration of endogenous pyridoxal phosphate, which remained unaltered in hippocampus following zinc administration (Itoh, and Ebadi, 1982a). The administration of a large doses of zinc chloride upto 100 mg/kg) either intravenously or intraperitoneal produced neither convulsive seizure nor other behavioural abnormalities.

The concentrations of zinc in 14 regions of rat brain including the hippocampus, were often higher (Ebadi, et al, 1981) than the administered doses producing convulsive seizures.

Proteins that bind zinc may be classified into at least three major groups consisting of:

a) Metalloenzymes
b) Metallothioneins
c) Metalloproteins

Henkins, et al, (1975) purified a zinc protein, gustin. It is thought to play a definite function in taste since its amount is lower in patients with hypogeusia.

1.15 Zinc Binding in Mammalian CNS:

The administration of 7.5 mg zinc/kg. body weight, as zinc sulfate, increased the concentration of zinc-binding protein in liver with the peak activity seen 6 hours after its administration. The value remained above control level for more than 48 hours. Unlike that in the liver, the administration of zinc sulfate did not increases the zinc binding proteins in any areas of brain, including hippocampus, cerebral cortex and basal ganglia.

In addition to donating zinc to an extensive number of apometalloenzymes and to protecting numerous sulfhyrl containing enzymes, the zinc binding proteins by maintaining the steady-state concentration of zinc, may play a decisive role in maintaining the functional integrity of synaptic membrane sympatic events and physiological receptor

sites including γ-aminobutyric acid (GABA). Smart and Constanti (1982) have shown that zinc depressed the GABA - evoked conductance increase. GABA applied to the dendrites of hippocampal pyramidal cells induces partial depolarization of the cells, in contrast to the usual hyperpolarizing response when GABA is applied to the cell bodies (Johnsen, Laursen 1981; Djorup, Jahsen Laurel 1981).

The high concentration of zinc in hippocampus mossy fibre boutons (Haug 1967) has given rise to numerous investigation of the role of zinc in hippocampal function, particularly with regard to mossy fibre synaptic transmission (Haug 1975).

In rats, development of hippocampus and accumulation of zinc into the hippocampal mossy fibres occurs late prenataly and during the first 3 weeks postuatally. The zinc concentration in the hippocampus was higher than in most other neural areas, excluding the cerebellum.

Evidence from electron microscopic studies has further indicated that the mossy fibre zinc is concentrated selectively within the gaint terminals of the mossy fibre axons (Haug 1967). Mossy fibre axons of hippocampus contain only 10 % of total hippocampal zinc (Frederickson et al, 1983).

1.16 Regional Distribution of Endogenous Zinc in Brain:

Zinc is the fourth most abundant ion in the brain (Donaldson et al 1973). In general, concentration is gray matter is twice as high as that in white matter (Harrison et al 1968).

The CA3 area of hippocampus contains more zinc per unit weight than either CA1 or whole hippocampus (Danscher et al 1976). More than half of the total hippocampus zinc is found in the nuclear pellet, where large boutons would be expected to sediment.

1.17 Analysis of the subcellular distribution of zinc:

The analysis of the subcellular distribution of zinc suggest that the metal could be present in several compartments free ions and those bound with different affinities to protein, metalloenzymes or other ligands. 20 % of hippocampal zinc is either free as ions or is bound to ligands of molecular weight less than 12.000 daltons. The remainder 80 % of zinc is apparently chelated or structurally bound to large molecules.

The results of these studies indicate that the relative binding of metals in brain differs from bound zinc albumin which is 30 % to 40 % tightly bound to an alpha-2-globulin (Wolff 1956).

Much of the ultrafiltrable serum zinc is bound to several amino acids: histidine, glutamine, threonine, cysteine, and lysine (Prasad 1979). About 2 % of loosely bound zinc is associated with cysteine and histidine.

1.18 Hippocampus and under nutrition:

Recent evidence showing alteration in spatial memory due to period of undernutrition during early life has implicated the hippocampus as one of the brain centers that may be particularly adversely affected. These results indicate that the rat dentate gyrus is vulnerable to undernutrition even during the post-weaning period and that a lengthy period of undernutrition can alter the developmental growth curve for synapse neuron ratio (Ahmed et al 1987). It has been a common observation that the cerebellum is particularly vulnerable to period of undernutrition during early life (Bedi 80ab). It has been suggested that this may be because granule cells which form the vast majority of cerebellar neurons develop postnatally whereas neurons in other brain region develop before birth.

Another brain region that also has neurons that develop postnatally is the hippocampus formation. In this region it has been estimated, from autoradiographic studies that about 80 % of the dentate gyrus granule cell arise after birth (Angevine '65; Schlessinger et al, 1975). Indeed some studies in the rat (Bayer et al 1982) have shown that granule cells of the rat dentate gyrus continues to be produced well into adulthood. It can, therefore, be postulated that the dentate gyrus like the cerebellum should also be markedly affected by a period of under-nutrition during early postnatal life.

The rat hippocampus is thought to be involved in the control of spatial memory (O' keefe and Nedel '78). Recent behavioural studies have indicated a possibility of deficits a spatial memory performance induced by a period of under-nutrition (Jordan et al '81, '82), though these findings have been disputed (Hall, '83).

West and Anderson (80) have published data on the synapse neuron's ratio of the dentate gyrus of normal rats and mice. They attempted to relate the number of granule cells in the granular layer to the number of synapses in the molecular layer.

Ahmed, et al, (1987) have shown that the under nutrition for an extended period during early perinatal life can cause alterations in the developmental curves for the

synapse-granule cell neuron ratio in the dorsal lip of the rat dentate gyrus. The changes produced could not be reversed by nutritional rehabilitation, at least in those animals undernourished from birth to 150 days of age. It is interesting to note that these latter changes occured despite the fact that the nutritional rehabilitation in the present experiments removed the deficits in fore-brain weight observed in the rats killed immediately after the period of under nutrition.

Age-related changes in our well-fed control rats. These show that in these animals the synapse neuron ratio increased with age to reach its peak at about 75 days of age. It seems that initially more synapses per neuron are produced than required in later life. Under nutrition from birth to 75 days of age did result in substantial (approximately 28 %) deficit in the synapse-neuron ratio when compared with age-matched controls. Therefore, it is possible that under-nutrition at first causes a reduction in the development of dendritic tree but that there comes a point when continued under-nutrition starts to cause an actual loss (whether by cell death or lack of normal neurogenesis, is not relevant) of a significant number of dentate granule cells.

1.19 Histo-chemical staining considerations :

1.19.1 Dithizone Staining :

Dithzone stains a variety of brain region differently. A substance, such as dithizone, which has a relatively high affinity for zinc (Honaker 1962; Math, 1964) and turns a specific colour (pink to red) upto binding with the metal, could have two histochemical uses.

1.19.2 Localization of zinc and quantitation of zinc :

Dithizone-stains tissue at relatively high magnification, in the form of dense, granules or "grains", which are distributed unevenly through the tissue. However, the careful work by Logothetopolous et al (1961) suggests that the granules are artifacts. Dithizone staining in the mossy fibre region seems entirely to be zinc dithizonate, and that the same is probably true elsewhere in the brain. Since dithizone reaction blocks the Timm's reaction it was argued that the Timm's reaction must also depend specifically upon zinc. The possible use of direct assays to determine the zinc content of the silver grains formed by Timm's procedure was proposed as a definitive, but technically challenging approach to the problem why metals such as iron and copper (and possibly some cerebral stores of zinc) apparently do not stain in either histochemical procedure was discussed, and the suggestion that perhaps only

weakly bound ions react histochemically was offered.

The dithizone method is commonly used for histochemical demonstration of zinc, but dithizone (diphenylthiocarbazone) forms an insoluble coloured inner complex salt with number of heavy metals (Zn, Pb, Cu, Hg, Au, Cd).

1.19.3. Zinc-Zincon Reaction:

Zincon (2 carboxy - 2 hydroxy 5'sulfoformazyl-benzne) has recently been used for serum zinc determinations under very controlled conditions, Hasan (1977) 'in vivo zincon staining'. Zincon has also been successfully used as an indicator for the spectrophotometric determination of zinc content of water.

The zincon staining solution which is deep red, forms a blue complex with zinc ions, especially in alkaline solutions (pH 9-10). Cold knife or cold microtome sections are undoubtedly best for the demonstration of natural tissue zinc. The zinc complex is stable over pH range of 8.5-10 whereas the copper zincon complex is stable in the pH range 5-9.5. This difference in effect of pH permits the ready detection of zinc at pH 9.5-pH 10 (Hasan 1977). The dithizone method, which is commonly used for histochemical demonstration of zinc, is not so specific. A unique characteristic of zincon is its ability to diffuse uniformly to almost every part of the brain to react with zincon intravitally.

1.19.4 Sulphide silver method:

The sulphide silver method, which histochemically demonstrates a number of heavy transitinal and group IIb metals (Timm 1958 Voigt 1959), stains the neuropil of the mature hippocampal formation in discrete laminaecorresponding to the terminal field of the various afferent pathways. Staining pattern directly depends on the innervation of the different laminae.

Semithin section from hilum of the rat hippocampus stained with Timm's sulphide method,The mossy fibre terminals were stained in the form of large black particles (Frank and Gaarskjar 1978).

1.20 Nomenclature:

In this and the following study a terminology adopted from Blackstad (56) and Blackstad et al (70) is employed. Accordingly, the anterodorsal end of the hippocampal formation towards the septum is termed septal end and the anteroventral end is termed the temporal end. The most posterior part of the hippocampus is called the occipital bend. Here the dentate fascia displays its characteristic V-shape when seen in transverse section. Its two limbs, here called medial and lateral crest (Cresta) the space between the two limbs is called the hilus and is essentially identifical to CA4 of Lorente de No. ('34). The Ammon's

horn is divided into regio superior characterized by small pyramids and a regio inferior characterised by large pyramids the two region correspond to field CA1 and field CA2+ CA3 by Lorente No respectively only the Pyramids of field CA3 receive mossy fibres. In regio inferior "Proximal" means closer to the hilus and "distal" means closer to regio superior.

1.21 The dentate area:

The dentate area is traditionally sub-divided into three layers : an outer molecular layer, middle granular and an inner hilus.

The septal two thirds of the dentate fascia are V-shaped and the crest is well-defined but at the transition to temporal third the dentate fascia gradually become U-shaped, still further temporally it is almost semi-circular and the crest becomes indistinct. Close to the fascia fasciola cinera the lateral blade of the granular layer is reduced first in thickness (i.e. the number of cell layers decreases) and cell density then in length, and finally disappears. The medial blade however, persist almost unaltered over this distance. The level at which the lateral blade disappears can be defined accurately since the crest clearly demarcates it from the medial blade. This level is in "septal end" in

the most temporal part of the dentata fascia, the medial part of the granular layer is reduced in length and probably disappears and the Crest becomes so indistinct as to make the separation of the two blades dubious. Along with the disappearance of the medial granule cells, the hilus appears on the medial brain surface, covered only by the pia. Further temporally almost the entire medial aspect of the hilus is exposed "temporal" end. The distance from this level to the actual disappearance of granule cells measures 500-1000 µm. The granule cells are densely packed in the main septal part of the dentate, and the inner border of the cell layer adjoins the hilus along an edge-sharp line, where as the outer border towards the molecular layer is relatively uneven. The packing density of the cells declines near the temporal end and the inner border becomes uneven to the degree that the granule cells seem to single with the nerve cells in the hilus. Almost all the nerve cells in the hilus loose the characteristic intense staining of the cytoplasm on approaching the temporal end. In addition, the form of the cell bodies appears more circular in sections, and their size decreases while the nuclear-cytoplasmic ratio increases.

1.22 <u>The regio Inferior</u>:

The septalmost pyramidal cell bodies are relatively scattered and form, in the entire proximodistal extent of

regio inferior a wide layer of ill-defined borders one or two millimeter from the septal end the arrangement of the cells in the distal part of the regio inferior tends to be more regular forming a narrower layer. Further temporally this tendency progresses towards the hilus, but only 2-3 mm from the temporal end does the entire extent of regio inferior acquire this appearance. A little further temporally the pyramidal cells nearest the regio superior are more scattered and a considerable number of pyramidal cells occurs throughout the entire width of distal part of the stratum oriens in the proximal part the pyramidal cells are still regularly arranged. However, this temporal level the superficial border of the layer of pyramidal cells is well demarcated from the stratum radiatum in the entire extent of the regio-inferior. In addition, near the temporal end the size of the pyramidal cells is reduced and so is the staining intensity of their cytoplasma resulting in blurring of the border toward the regio-superior.

1.23 Organisation of the Mossy Fibre System:

The pyramidal layer of the regio inferior is broad and the cells within the layer are relatively widely spaced. Here the mossy fibre boutoms intrude deep in the layer. At more temporal levels the cells are tightly packed, the cell layer is narrower, and the boutons predominantly terminate suprapyramidally.

The length of granular layer is greatest septally, while the length of the mossy fibre layer is greatest temporally. The area of the hilus occupied by the mossy fibre boutons is large at most of the temporal levels and small at the septal levels, where as in CA3 the reverse is true, the greatest number of granule cells was found at the more septal levels. Conversely, the majority of the pyramidal cells in the hilus and in the regio inferior is situated temporally. The ratio of granule cells to pyramidal cells (hilus and regio inferior together) declines linearly from approximately one at temporal levels. Because the mossy fibres are distributed fairly transverse to the long axis of the hippocampus, the variation in the ratio of granule cell to pyramidal reflects a corresponding variation in the number of innervating to inervated cells within the mossy fibre system.

The commissural and ipsilateral connections from the hilus fasciae dentatae (CA4) and regio inferior (CA3/CA2) were found to terminate in different parts of the hippocampus. The hilus fascia dentatae gave rise to ipsilateral and commissuoral projections to the dentate area only. The regio inferior has ipsilateral and commissural projection to the Ammon's horn.

A specific termination pattern was found of the projection from regio inferior to stratum radiatum of both

the ipsilateral and contralateral regio superior (CA1) and regio inferior (CA2/CA3).

Each part of regio inferior projected to all parts of stratus radiation and oriens of the contralateral Ammon's horn.

Swanson and Gowan (77) after injection of tritiated amino acid into CA4 observed no labelled extrahippocampal projections. Further study indicatesthat there is no substantial projection from the hilus fasciae dentatae to the Ammon's horn and that the only major intrahippocampal connections of hilus are the bilateral projections to the dentatae area. This implies that the hilus neurons take part only in the local circuitries of the area dentatae themselves.

Since the hilus fascial dentatae has no major connection with Ammon's horn and the Ammon's horn does not project to the area dentata, the mossy fibres are the only major connection between the area dentata and the Ammon's horn. Hjorth-Simonsen (73) and Swanson and Cowan (77) described a unidirectional organization of the ipsilateral projection, regio-inferior supplying regio-superior and the Ammon's horn the subiculum. This investigation demonstrates one-way connection between area dentata and regio-inferior. It is striking and hardly not without important functional implications that the area dentata, the Ammon's horn, and the subiculum all receive an input directly from the entorhinal area and that each of these areas has a unidirectional

connection with the next in the direction from area dentata to the subiculum.

The mossy fibres are the axons of dentata granule cells. They synapse by giant boutons with the pyramidal cell dendrites of CA3 and CA4 (Cajal 1893, Blackstad et al, 70). The boutons show intense sulphide silver staining their axon (Haug et al 71).

The terminal area of the mossy fibres: The mossy fibres, a major intrinsic hippocampal pathway connecting the dentate granule cells with the pyramidal cell in CA4 and CA3.

In general, the suprapyramidal layer of mossy fibre boutons is widest in the main middle part of the regio inferior and tapers towards the hilus and towards the regio superior. In its septal one fourth to one third over the entire proximal extent of the field CA3, mossy fibre boutons are present and form a relatively wide layer. More temporally the layer gradually becomes more slender since the intrapyramidal boutons disappear, in a sequence from the distal to the proximal end of the CA3 and about 1500 µm from the temporal end they have almost totally disappeared. Further temporally still, the layer of mossy fibre boutons which is well defined septally, become blurred, especially towards the pyramidal layer, and distally a small amount of intrapyramidal boutons re-appear proximally, however,

virtually no boutons are present.

1.24 Zinc and Mossy Fibres:

The functional role of zinc in the mossy fibres has not yet been established, but the possibility exists that it is associated with the metabolism of the putative neurotransmitter glutamic acid either through the zinc metalloenzyme - glutamic acid dehydrogenase, or as stable zinc glutamate storage complex in the giant boutons (Crawford, Connor 1975). Considerable attention has been paid to the accumulation of zinc by the hippocampus in the first few weeks postnatally. Zinc accretion has been measured both by elemental analysis and autoradiagraphy and by various histochemical methods notably Timm's silver sulphide stain and intravital staining with dithizeone.

Autoradiographic studies suggest that zinc uptake takes place initially into granule cell soma and then probably passes by axoplasmis transport to the terminal boutons (Crawford, Connor 1973). Further studies are needed to establish the size and characteristics of the various hippocampal zinc pools and to determine whether the intense staining of the mossy fibres reflects genuinely

higher zinc levels or some histochemical peculiarity of reactive metals in that region.

Many questions concerning the accretion of zinc by the hippocampus remain unanswered little is known regarding the form in which zinc is stored in the mossy fibres, or indeed in other areas of the hippocampus, or of the respective function of these metal complexes work by (Haug et al 1971) concerning the anterograde degeneration of hippocampal mossy fibres following lensions in the fascia dentata.

In chronic alcoholics, low plasma zinc levels often accompany prolonged alcohol abuse McLardy(1975) has reported that zinc level are much reduced in hippocampus from alcoholic pateints and that the granular cell layer of the dentata gyrus is abnormally thin, suggesting zinc deficiency may be involved pathologically in alcohol related mental deterioration.Of particular relevance,in this connection is the report by Walker et al (1980) that in adult rats, chronic ethanol consumption led to loss of hippocampal pyramidal cells and granular cells in the dentata gyrus.

Also pertinent is the observation by West et al (1981) that the organization of mossy fibres in the hippocampus was significantly altered in rats exposed to alcohol prenatally, due to the appearance of an aberrant band of intrapyramidal fibres in hippocampal subfield CA3.

The pineal body too deserves special mention as the high zinc level in this organ may also be related to neuro transmission (Demmel 1982).

1.25 Zinc and Neurobiology:

The role of zinc in neurobiology has been discussed at two levels. Firstly, the need for zinc during cell division has been related to the developmental anomalies of the central nervous system seen in experimental animals and possibly in humans. Experimental evidence is presented drawing attention to the role of the zinc-dependent enzyme thymidine kinase for DNA synthesis in neural tissue. Secondly a possible functional role for zinc in the brain has been raised, especially in connection with the mossy fibre pathway of the hippocampus and other glutamergic system, where an involvement of zinc appearans possible in relation to metabolic or storage of neuro transmitter glutamic acid other metabolic events with which zinc is related and which may be of neurochemical significance include brain catecholamine levels enkephalin receptor binding and the concentration of circulating corticosteroids. Special mention is made of the effect of zinc deficiency on behaviour in experimental animals and of the possible parallel condition seen in human.

1.26 Function of Hippocampus:

The function of the hippocampal formation is not fully known. Experimental findings indicate that it is involved in a wide diversity of partial functions. A theory that might explain the function of the hippocampus as a whole on the basis of these partial functions has yet to be advanced. The hippocampus seems to exert a strong influence in modulation of aggressive behaviour patterns (of hypothalmic origin). Stimulation of temporal end facilitates the development of aggressive reactions, and stimulation of septal end has the opposite effect. This functional difference may be related to the unequal projection of these areas to the septum. Lesion of the hippocampus in experimental animals causes a tendency to persist in a given behaviour pattern although the original reason for this behaviour has disappeared. These lesions cannot suppress a trained behaviour pattern.

It has recnelty been established that the pyramidal cells of the hippocampus are able to bind certain hormones (estradiol, corticosterone) in a relatively high concentration. Thus the hippocampus is believed to be capable of measuring the serum hormone level and transmitting information to the hypothalamohypophyseal system via the precomissural fornix and the septohypothalamic projections. This feed-back machanism could contribute to the regulation

of the release of these hormones. Clinical findings are suggestive of a mnemonic function (short-term memory). Bilateral ablation of the based temporal cortex and hippocampus causes amnesia for recent events. The patient is quite capable of following conversation but loses the drift of it as soon as a subject is changed.

Material and Methods

2- MATERIAL AND METHODS

2.1 Animals:

Healthy male Charles Foster strain albino rats weighing 150-200 g were obtained from the central animal house, J.N. Medical College, Aligarh, U.P., India. Females were excluded from this study because of their cyclic hormonal variation.

2.2 Preparation of Animals:

The animals were kept for one month at approximately constant room temperature ($20^{o}C$ - $25^{o}C$), and were fed standard pellet diet.

2.3 Method of study and number of animals used:

The number of animals for quantitative estimation of zinc, copper, aluminium and manganese and histological and histochemical studies is given in table 1.

Table - 1: The number of animals used for quantitative estimation of zinc, copper, aluminium and manganese and for histological and histochemical studies.

	Method of Study	Number of animal used
1.	Quantitative estimation of zinc, copper, aluminium and manganese	8
2.	Histological studies	
	a) Paraffin section	4
	b) Plastic section	4
3.	Histochemical studies	
	a) Zinc-zincon reaction	2
	b) Alkaline phosphatase	4

2.4 Procedure for killing animals:

For quantitative estimation and histological and histochemical studies the animals were divided into two groups:

I) Group 1

The group one comprised of 12 animals.8 animals were used for quantitative estimation of zinc, copper, aluminium and manganese.4 animals were used for alkaline phosphatase demonstration. They were killed by cervical dislocation.

II) Group 2

The second group comprised of 10 animals. 4 were used for histological studies with a paraffin embedment and 4 were used for histological studies with araldite embedment. 2 were used for demonstration of zinc in the form of zinc-zincon reaction. The animals were deeply anesthetized by intraperitoneal injection of 35 mg/kg body weight of pentobarbital sodium and were killed by intracardiac perfusion of fixatives.

2.4.1 Perfusion fixation technique:

Technique for fixation of the brain by perfusion has been described in detail by Hasan et al. (1977). The thorax of anaesthetized animal was opened by an incision starting at the base of xiphoid process and extending along the median line of the sternum to the jugular notch avoiding the internal mammary artery that runs close to the sternum. The opening of the thorax was then widened by cutting symmetrically along an intercostal space on each side, starting from xiphoid process, the flaps of the thoracic wall formed this way were clamped and rolled upward. 18 guage needle was introduced into ascending aorta through the left ventricle. The right atrium was widely opened and the perfusion started at a pressure of about 5 feet of water after initial vascular perfusion with buffered saline solution.

2.4.2. Cervical dislocation:

The animals were grasped at their neck near the base of the skull, with the thumb and forefinger of one hand and hind limb and tail with other hand. A swift but controlled motion separated the cervical vertebrae from the base of the skull. This resulted in instantaneous loss of consciousness and loss of vital sign's within less than a minute.

2.5 Exposure of the brain and its removal;

The skin covering back of the skull was reflected and the skeletal elements protecting the brain were removed. The brain was exposed and was gently detached from the base of the skull and taken out.

2.6 Dissection of different parts of brain:

For paraffin section and histochemical demonstration of zinc and alkaline phosphatase the brain was sliced in 3 mm thick sections. They were immersed in the remaining perfusion fluid.

For plastic (araldite) sections the required amount of frontal cortex, cerebellum, medulla oblongata and hippocampus were dissected out.

2.6.1 Dissection of the hippocampus:

The term hippocampus will refer in this dissertation to the pyramidal field's of Ammon horn. The overlying cortex was removed and the total hippocampus was carefully taken out with fine forceps and sectioned perpendicular to the long axis into three equal pieces. The rostral extent of the hippocampus will be called the septal pole and the extreme opposite end the temporal pole. The portion in between these two pole will be termed as middle portion.

The areas CA_4, CA_3, CA_2 were identified with the help of dissecting microscope and were carefully removed with sharp blades.

2.7 Paraffin sections for light microscopic study:

2.7.1 Dehydration of tissue:

The tissues contain large amounts of water both intracellularly and extracellularly. This water must be removed, so that it may be replaced by wax. Dehydration is best accomplished by the use of graded alcohols beginning with 50 % alcohol.

2.7.2 Dehydration procedure:

50 % alcohol two changes, each of 1 hour
70 % alcohol two changes, each of 30 minutes
95 % alcohol two changes, each of 30 minutes
Absolute alcohol two changes, each of 10 minutes.

2.7.3 Clearing (Dealcoholization):

This was to be accomplished so that alcohol in the tissue is replaced by fluid which will dissolve the wax with which the tissue must be impregnated. Tissue was placed in a conical glass containing xylene approximately 10 times the volume of the tissue and was vigrorously shaken till the tissue became clear.

2.7.4 Impregnation and embedding:

The tissues having been completely dehydrated and cleared were impregnated with paraffin wax by immersion in a succession of molten wax baths. Later it was processed by hand for 3 hours in 3 changes of wax.

2.7.5 Blocking out moulds:

The L-shaped thick copper mould was placed on glass plate and was adjusted to give a suitable block size.

2.7.6 Procedure:

The wax was kept molten in an oven maintained at 2°C higher temperature than the melting point of the wax used (60°C). The mould was thereafter filled with wax to within a few mm of its top. The tissue was picked up by a pair of

blunt forceps which had been slightly heated and the tissue was placed in the mould of molten wax. The side of the tissue from which it was desired to take sections was placed face down. The block was embedded and as soon as the wax had become partly solid, the cup was placed in basin of cold water (10 to 18^{o}C).

2.7.7 Trimming of blocks:

When the blocks were hardened in cold water they were removed and the excess wax cut off in thin slices, so that the block forms a truncated pyramid, At least 2 mm of wax should surround the tissue block.

2.7.8 Attachment of the block to block holder:

The bottom of the wax block was placed on the heated spatula and as soon as the bottom of the wax block melted the wax block was pressed down firmly and allowed to harden.

2.7.9 Section cutting:

Spencer's rotatory microtome was set for the desired thickness of (6 um) sections. The section were obtained slowly with a very even stroke.

2.7.10 Obtaining ribbons of sections:

Upper and lower surface of the block was made parallel and straight "ribbon" of sections was obtained. The ribbons were floated on the surface of the warm water in water-bath; When the sections were flattened out a clean slide was immersed nearly vertically in the water bath. The surface of the slide coated with adhesive was then gently approximated to the end of the ribbon. The slide was gently raised with an even motion and as it touched the edge of the section, the section was adhered to it and the slide was drawn upward. The excess fluid on slides was then allowed to drain out at an angle of about 60 to 65 degrees for approximately 2 to 5 minutes.

2.7.11 Drying sections on slides:

Slides were kept in an oven at 56 to 60^{o}C for 2 hours.

2.8 Method of Preparation of fixatives:

2.5 % Paraformuldehyde, 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) was used for histological studies.

2.8.1 Solutions:

Solution A)	Paraformoldehyde	7.5 g
	Double Distilled Water (DDW)	100 cc

This mixture was heated to 60°C-70°C with constant stirring and drop by drop 1 N NaOH (sodium hydroxide) was added till the solution became clear. It was then cooled under running tap water. To this clear solution, 7.5 cc glutaraldehyde was added and the final volume was brought to 150 cc with the help of DDW, the pH of this solution was adjusted to 7.3 with the help of 1 N Hcl solution.

Solution B)	NaH_2PO_4 $2H_2O$	1.5601 g
	DDW	50 ml
Solution C)	Na_2HPO_4 $2H_2O$	5.3448 g
	DDW	150 ml
Solution D)	Solution B	30 ml
	Solution C	125 ml
	pH of this solution was	7.4

Mixture solution A with solution D and adjust the pH to 7.3 with the help of remaining solution B and C.

2.9 Histological staining methods for paraffin sections

2.9.1 Cresyl violet and luxol fast blue staining

2.9.1.1 Reagent:

I)	Luxol fast blue	0.5 g
	Methylated spirit	475 ml
	Distilled water	25 ml
	Acetic acid (10 %)	2.5 ml
II)	Aqueous lithium carbonate	(0.05 %)
III)	Cresyl violet	100 mg
	Distilled water	100 ml

The sections were dewaxed in xylene and brought to 90 % alcohol and then stained in luxol blue fast solution at room temperature over night (or at 56°C for 4-5 hours), rinsed in alcohol (to remove excess of stain), washed in distilled water, treated with lithium carbonate solution for 5 to 10 seconds and rinsed several times with 70 % alcohol until the gray matter in the sections became colourless. Sometimes when 70 % alcohol failed to decolorize the gray matter, the sections were returned to lithium carbonate (5 to 10 seconds) and then rinsed again in 70 % alcohol after the gray matter appeared nearly colourless and the sections were washed in running tap water and counterstained with cresyl violet for 10 minutes at 37°C, washed in distilled water, rinsed in 70 % alcohol and then in 95 % alcohol, cleared in xylene and mounted in DPX.

2.9.1.1.1 Plastic (araldite) sections for light microscopic studies:

The steps of fixation and dehydration for plastic sections are similar to those used for paraffin section.

2.9.1.1.2 Reagents:

I) Uranyl acetate solution 0.5 %

Uranyl acetate	0.5 g
DDW	100 ml

Clear solution was prepared and left overnight to settle down.

II) Working uranyl acetate solution:

With the help of a pipette required volume of uranyl acetate was taken, precaution was taken not to stirr up the solution.

III) Buffered osmium tetroxide (2 %):

One gram capsule of osmium tetroxide (OsO_4) was carefully broken immersed, in a jar containing 50 ml of 0.1 m phosphate buffer and was left overnight to dissolve and thereafter stored in a refirgerator.

IV) Working buffered osmium tetroxide (1 %):

The stock solution (2 %) of the osmium tetroxide was diluted with the help of 0.1 m phosphate buffer to 1 % buffered osmium tetroxide.

V) Plastic embedding media:

Suggested by Agar Aids for Electron microscopy stock mixture.

Araldite Cy 212	10.0 ml
DDSA	10.0 ml
BDMA	0.4 ml

Softer blocks may be obtained by adding a small amount of plasticiser, dibutyl phthalate (e.g. 1,0 ml to the stock embedding medium). For harder blocks, replace 1.0 ml of the DDSA with 1.0 ml of the hardener, methylnadic anhydride (MNA). Complete mixing of the componenets of epoxy resin embedding media is very important and is facilitated if Araldite resin and the DDSA hardener and a graduate cylinder and a conical flask are warmed to 60°C before mixing. Measure equal parts (by volume) of thewarm resin and hardener (from their separate containers) into the warm graduated cylinder and pour them immediately into the warm conical flask. Shake the mixture gently by hand rotation. Mixing will be complete within a few minutes if the components and the mixing vessels have been pre-warmed. Then add the BDMA (0.4 ml for every 10 ml of araldite and 10 ml of DDSA), and continue shaking by hand for a further minute or two.

2.9.1.1.3 Procedures:

The brain of the animal was removed between 3-4 hours after killing of the animal by intracardiac perfusion of 0.1 M phosphate buffer fixative. Parts of the brain already fixed by perfusion method were dissected out in small pieces and were washed with 0.1 M phosphate buffer and were stained with uranylacetate for 2 hours and washed with 0.1 M phosphate buffer and post-fixed in 1 % osmium tetroxide for 2 hours. After osmication the specimens were washed in 3 changes of 0.1 M phosphate buffer and were dehydrated with graded strengths of ethanol according to the following schedule.

30 %	ethanol	10 minutes
70 %	,,	10 minutes
80 %	,,	10 minutes
90 %	,,	10 minutes
95 %	,,	10 minutes
Absolute	,, I	10 minutes
Absolute	,, II	10 minutes

After complete dehydration the pieces were embedded at room temperature in the working plastic mixture according to following schedule.

2:1 absolute alcohol and plastic mixture for 60 minutes
1:2 absolute alcohol and plastic mixture for 60 minutes
Pure plastic mixture for 60 minutes

The tissues were transfered to fresh plastic mixture in the plastic mould and kept overnight in an incubator at 60°C for polymerization of plastic. The blocks were ready for section-cutting after two days.

2.9.2 Cutting of blocks:

After trimming of blocks, 0.5 um to 1 um semi-thin sections were cut on a LKB-ultratome using glass knives made with LKB knife maker. The sections were stained with toluidine blue solution and photomicrographs were obtained.

2.9.1.1.4 Histological staining methods for plastic sections

2.9.1.1.4.1 Toluidin blue

2.9.1.1.4.2 Reagent

Toluidine blue	0.1 g
2.5 % Aqueous sodium carbonate	100 ml

Sections were floated on freshly prepared toluidine blue solution for 2 hours. Excess of solution was washed off with 80 % ethnol and after dehydration cleared in xylene and mounted in DPX.

2.10 Histochemical staining methods:

2.10.1 Hasan's Perfusion staining method (1977):

2.10.1.1 Staining Solution:

1) Dissolve 0.13 g of 2 carboxy-2'hydroxy-5' sulfo-formazyl benzene (zincon) in 2 ml 1 N NaOH and dilute to 100 ml with redistilled water.

2) Buffer. Dilute 213 ml 1 N NaOH to 600 ml with redistilled water. Dissolve 37.3 g Kcl and 31 g H_3BO_3 in the solution and dilute to 1.1 litre with redistilled water.

3) Perfusion fluid. To 100 ml of solution 1 add 200 ml solution 2 (or enough to bring the pH to 10.0).

2.10.1.2 Perfusion apparatus:

An intra venous infusion set was used consisting of a bottle a drop chamber and 6-7 feet of rubber tubing supplied with squeeze type flow regulator, the tubing was connected to a cannula.

2.10.1.3 Procedure of perfusion staining:

Preparation of animal for the perfusion staining was similar to perfusion fixation technique (vide supra). After the heart was exposed 18 gauge needle was inserted into the ascending aorta through the left ventricle. The rate

of perfusion was adjusted to 8 ml/min. Perfusion was discontinued after 10-15 minutes when oozing of fluid appeared from the cut made in the upper lip. After the perfusion the brain was removed and was cut into slices and immersed in the perfusion solution for 10 minutes at $4^{o}C$ in a refrigerator.

2.10.1.4 Procedure for cryostat sectioning:

The slices of the rat brain were frozen on the freezing stage of a cold microtome. The blocks (slices) were 2 to 4 mm, thick so as to minimize the risk of metal object disc stricking the knife edge. The section were cut at 20 µm thickness with slow even motion. A clean slide was carefully lowered onto the section, in practice one edge of the glass slide was rested on the knife surface about 1 inch beyond the section and the other end was lowered gently until it was about 1/2 to 1 inch from the knife face. The section was automatically transferred from cold knife to the relatively warm slide. Sections were air dried and examined under light microscope and the photomicrographs of the appropriate regions were obtained.

2.10.2 Gomori Method (1952) for demonstrating alkaline phosphatase activity.

2.10.2.1 Staining Solution:

I)	3 % (0.1 M) solution of sodium glycerophosphate	5-10	ml
	2 % (0.2 M) solution of $CaCl_2$	20-26	ml
	10% (0.5 M) solution of $MgCl_2$	0.5	ml
	Sodium barbital power	0.5-1.0	g
	Distilled water to make	50	ml
II)	2 % cobalt sulphate solution	100	ml
III)	0.3 % solution of yellow ammonium sulfide	100	ml

2.10.2.2 Procedure:

2.10.2.2.1 Fixation:

3 mm thick slices of brain tissue were fixed in 80 % ethyl alcohol, cooled to 1°C for 12 hours. Subsequent dehydration was carried out in refrigerator.

2.10.2.2.2 Clearing:

Remove the alcohol with 2 changes of chloroform 15 minutes each. The rest of procedure was similar to that used for paraffin section (see

2.10.2.2.3 Procedure for staining:

The paraffin sections were dewaxed in xylene and hydrated in the customary manner and incubated at 37°C for 4 hours

in the solution I then washed in tap water for about 1 minute and immersed in solution II for 15 minutes. It was then washed in tap water for 2 minutes and immersed in solution III for 60 minutes and then washed with tap water dehydrated, cleared and mounted in DPX.

2.11 Procedure for quantitative estimation of zinc, copper, aluminium and Manganese:

2.11.1 Washing of the glassware:

All glassware used was soaked in 10 per cent nitric acid rinsed with deionized water (metals free water) to avoid contamination with metals.

2.11.2 Dissection of different parts of the brain:

The brain was removed rapidly, wiped free of adhering blood. The different regions were dissected out carefully and weighed separately. During the dissection and subsequent chemical manipulation care was taken to minimize contamination with extraneous metals. Thus, the brain was removed with the leptomenninges intact and transferred to a glass dish. The outer surface of the brain was then washed with deionized water, the leptomeninges stripped from the cortical areas to be examined and the required pieces of brain were dissected out with stainless steel instrument.

2.11.3 Acid digestion procedure:

Frontal cortex, hippocampus, cerebellum and medulla oblongata of the animals were digested in 7 ml. acid mixture containing concentrated nitric acid and perchloric acid in ratio of 5 to 1. After complete digestion the volume was made upto 8 ml. Metals in this solution were estimated by a Perkin-Elmer nodel 303 atomic absorption spectrophotometer.

Observation

3- OBSERVATION

3.1 Histological Examination of Brain:

3.1.1 Luxol Blue Fast and Cresyl Violet Staining:

Luxol blue fast and cresyl violet staining of the hippocampus (Fig. 1) reveal normal histological structure of the hippocampus. The nuclei of the hippocampal cells are stained violet in colour while mossy fibre are stained blue in colour. The molecular layer, the fascia dentata and the hilum (CA4) are seen.

3.2 Toluidin Blue Staining:

Semi-thin araldite plastic section (0.5 µm) stained with toluidin blue was used for the study of neurones and mossy fibre boutons in the rat hippocampus.(Fig. 2) Toluidin blue staining of (0.5 µm) plastic section of the septal part of rat hippocampus reveals the normal structure of neuropiles. The granule cells are densely packed and show a uniform circular outline and large nucleus with scanty cytoplasm around it, and a nucleolus. The granule cell layer of the hippocampus are clearly demarcated from the

deeper layer of pyramidal cells. Pyramidal cell shows light and dark staining properties with toluidin blue staining which indicates the presence of dense and scanty cytoplasm in different pyramidal cells of the rat hippocampus. The pyramidal cells are relatively widely spaced. Here the mossy fibre boutons intrude deep into this layer. The area of the hilum, occupied by the mossy fibre boutons The mossy fibre boutons are seen in the form of large black particles in this region.

The mossy fibres are the axons of dentate granule cells. They synapse with giant boutons with the pyramidal cell dendrites of CA3 and CA4. Autoradiographic studies suggest that zinc uptake takes place initially into granule cell and then probably passes by axoplasmic transport to the terminal boutons (Crawford, Conner 1973). Although mossy fibre terminal stain by various histochemical methods notably Timm's silver sulfide stains intravital staining with dithizone but doubts regarding the intense staining peculiarity of the mossy fibre exist whether they genuinely reflect higher level of zinc or some histochemical peculiarity of reactive cells in that region.

The report of Frank & Gaarskyar (1978) in the semithin section of the rat hippocampus stained with Timm's sulfide the mossy fibre boutons in the CA4 were stained in the form of large black particles. This similarity of staining of mossy fibre terminal in semithin section

stained with toluidin blue and Timm's sulfide may be due to the large size of these mossy fibre terminal which can be visualised with the help of light microscope and it does not reflect any special peculiarity of zinc staining in this region.

Little is known regarding the form in which zinc is stored in the mossy fibre or indeed in other areas of hippocampus of the respective function of these metal complexes (Haug et al, 1971).

3.3 Zinc-Zincon reaction:

Histochemical observation with zinc-zincon reaction (Fig.3) hippocampus (Fig. 4) cerebellum (Fig. 5) caudate and putamen nucleus reveals a uniform zinc-zincon reaction product in rat brain. The zinc-zincon reaction in the nucleus produces brown coloured nuclei, while in the cytoplasm and to the nerve fibre it imparts a light blue colour reaction. The zinc-zincon staining solution which is deep red forms a blue colour complex with zinc ions especially in alkaline solution (pH 9-10). The difference in zinc-zincon reaction in the nucleus and cytoplasm may be due to the fact that zinc in the nucleus is not in the free form and is bound to enzymes such as thymidine kinase, RNA and DNA polymerase, hence the physiological state of zinc in the nucleus and cytoplasm is markedly different.

Using a very precise method of atomic absorption spectroscopy, zinc, copper, manganese and aluminium concentration in

the cerebrum, hippocampus, cerebellum and medulla were measured. (Table 2). The highest concentration of zinc was found to be present in the hippocampus.

In 20 µm cryostat sections zinc-zincon reaction in the neuropil was uniform staining reaction and it does not detect different zinc concentration on the various region of the rat brain, but the specificity of this staining reaction is that zincon only react with zinc to form blue colour product of zinc zincon.

3.4 Alkaline Phosphatase reaction:

Histochemical demonstration of alkaline phosphatase in the hippocampus (Fig. 6) and cerebellum (Fig. 7) by (Gomori Tech) reveals sites of alkaline phosphatase activity in the form of black and gray colour granules in the rat brain.

Table - 2: Rat Brain Zinc, Copper, Manganese and Aluminium concentration in ug/g fresh tissue

Sample of tissue	Zn	Cu	Mn	Al
Cerebrum	52.12±07	6.58±0.3	5.36±0.3	35.9 ±0.6
Hippocampus	81.73± 1	9.36±0.7	9.8 ± 06	53.36± 1
Cerebellum	49.02±04	4.97±0.6	5.58±0.5	27.44±0.4
Medulla	57.84±0.5	5.89±0.9	6.9 ±0.5	36.59±0.7

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Illustrations

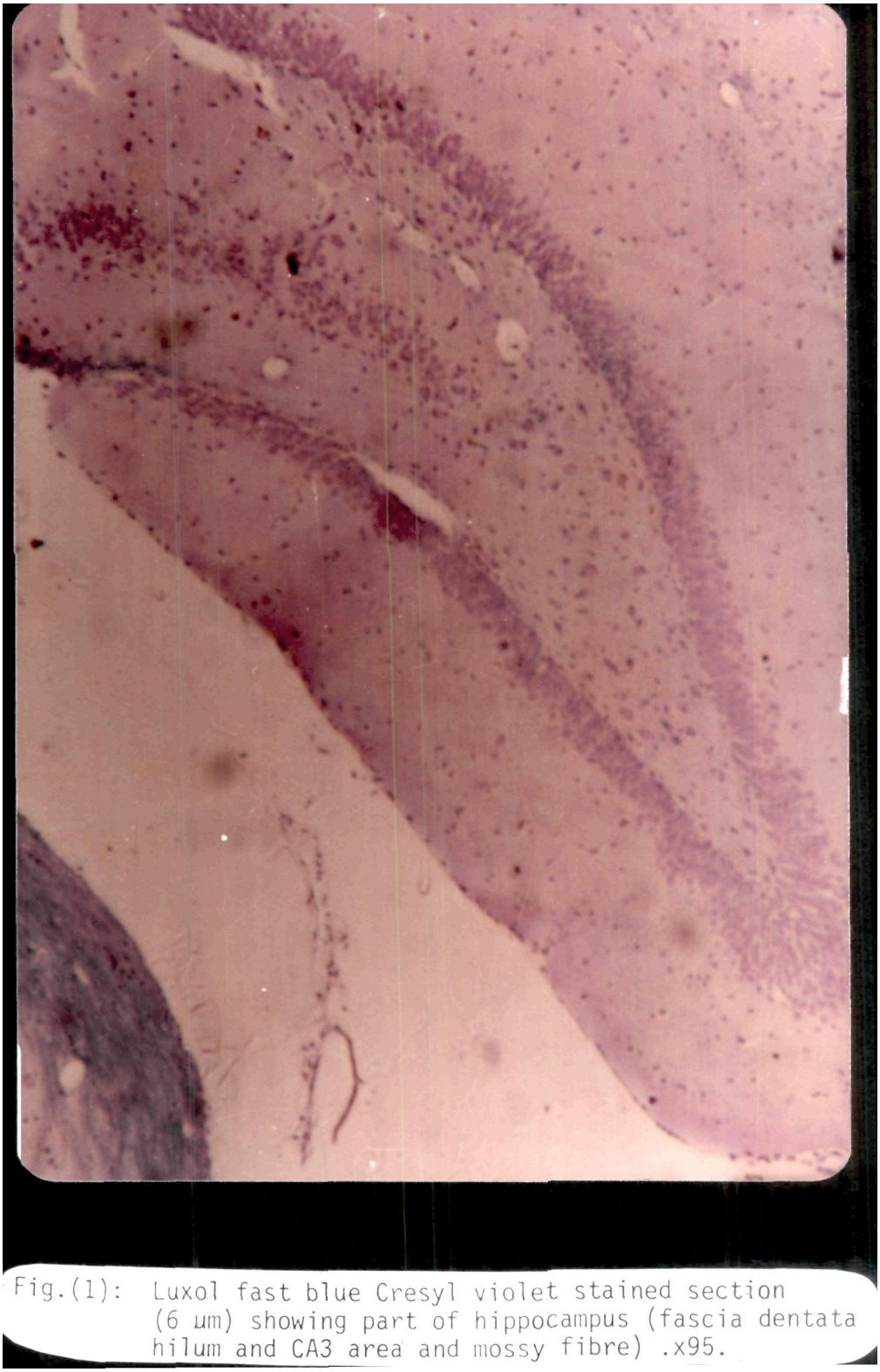

Fig.(1): Luxol fast blue Cresyl violet stained section (6 µm) showing part of hippocampus (fascia dentata hilum and CA3 area and mossy fibre) .x95.

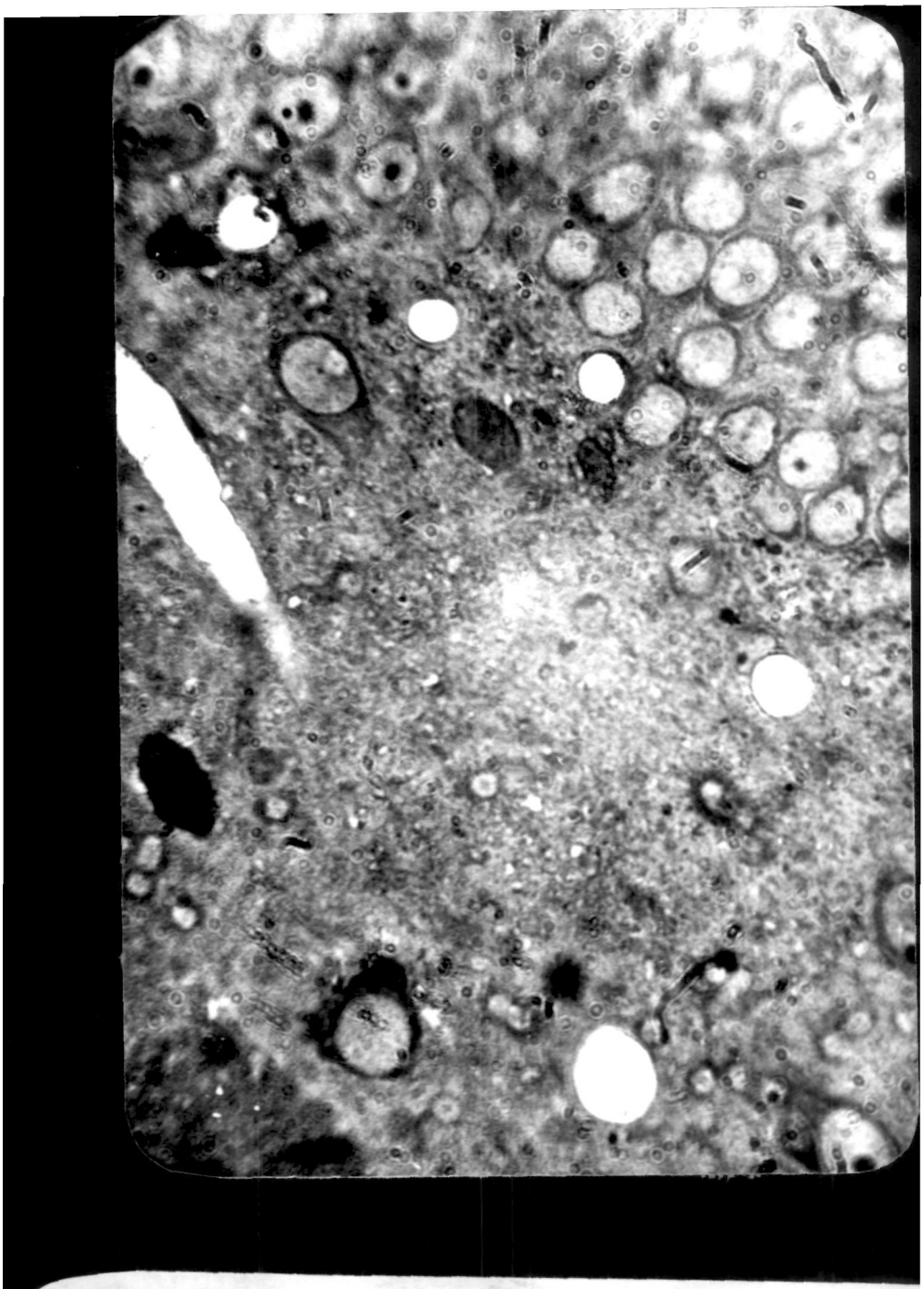

Fig.(2): Semithin toluidine blue stained section (0.5 μm) showing part of dentata area and the hilum of the hippocampus. Note the density of the mossy fibre boutons (large black particles) .x950.

Fig.(3): Cryostat section (20 μm) showing part of hippocampus (CA2). Note the zinc-zincon reaction in the nucleus (Brown colour and in the cytoplasm and nerve fibre light blue colour. x95

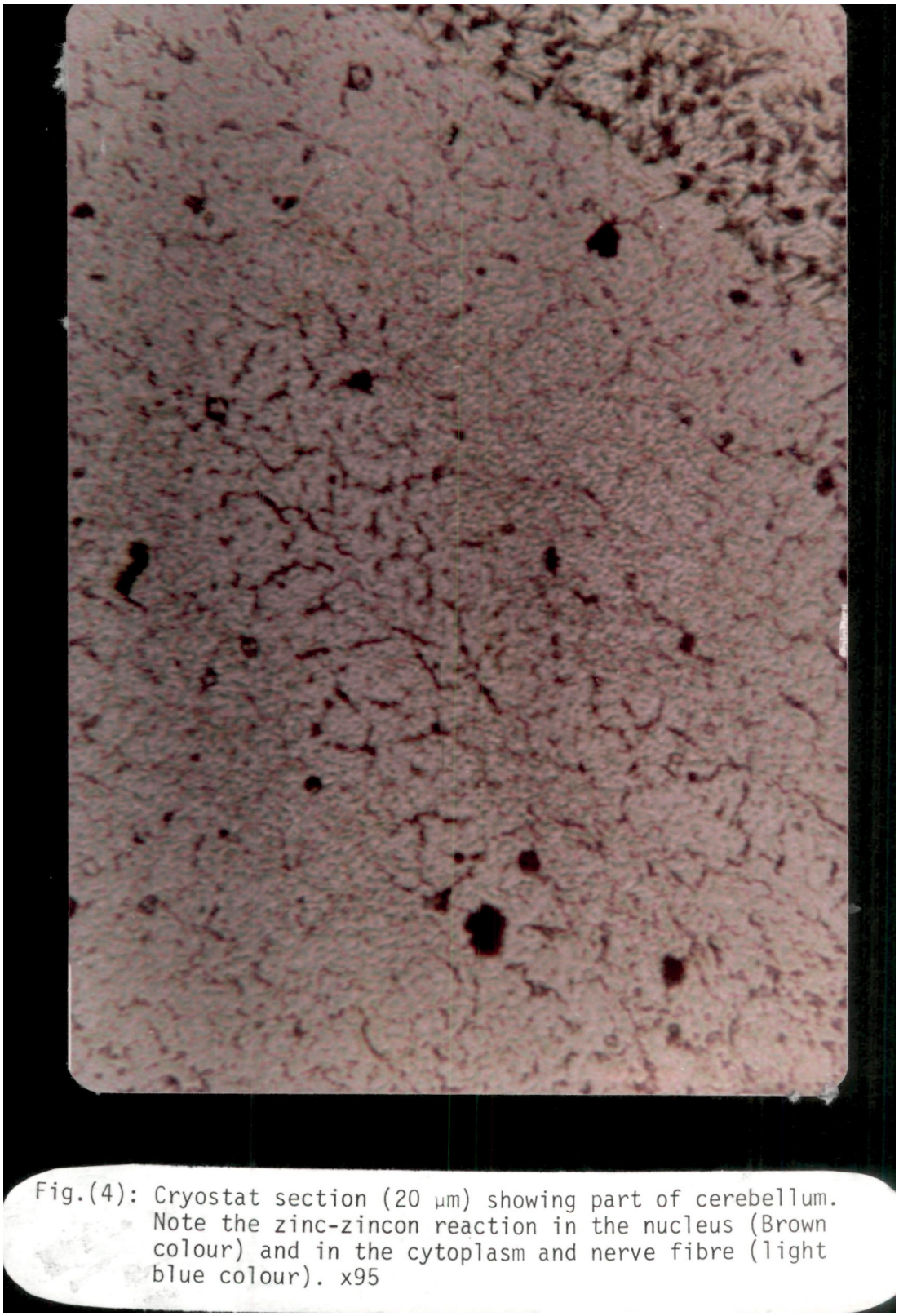

Fig.(4): Cryostat section (20 µm) showing part of cerebellum. Note the zinc-zincon reaction in the nucleus (Brown colour) and in the cytoplasm and nerve fibre (light blue colour). x95

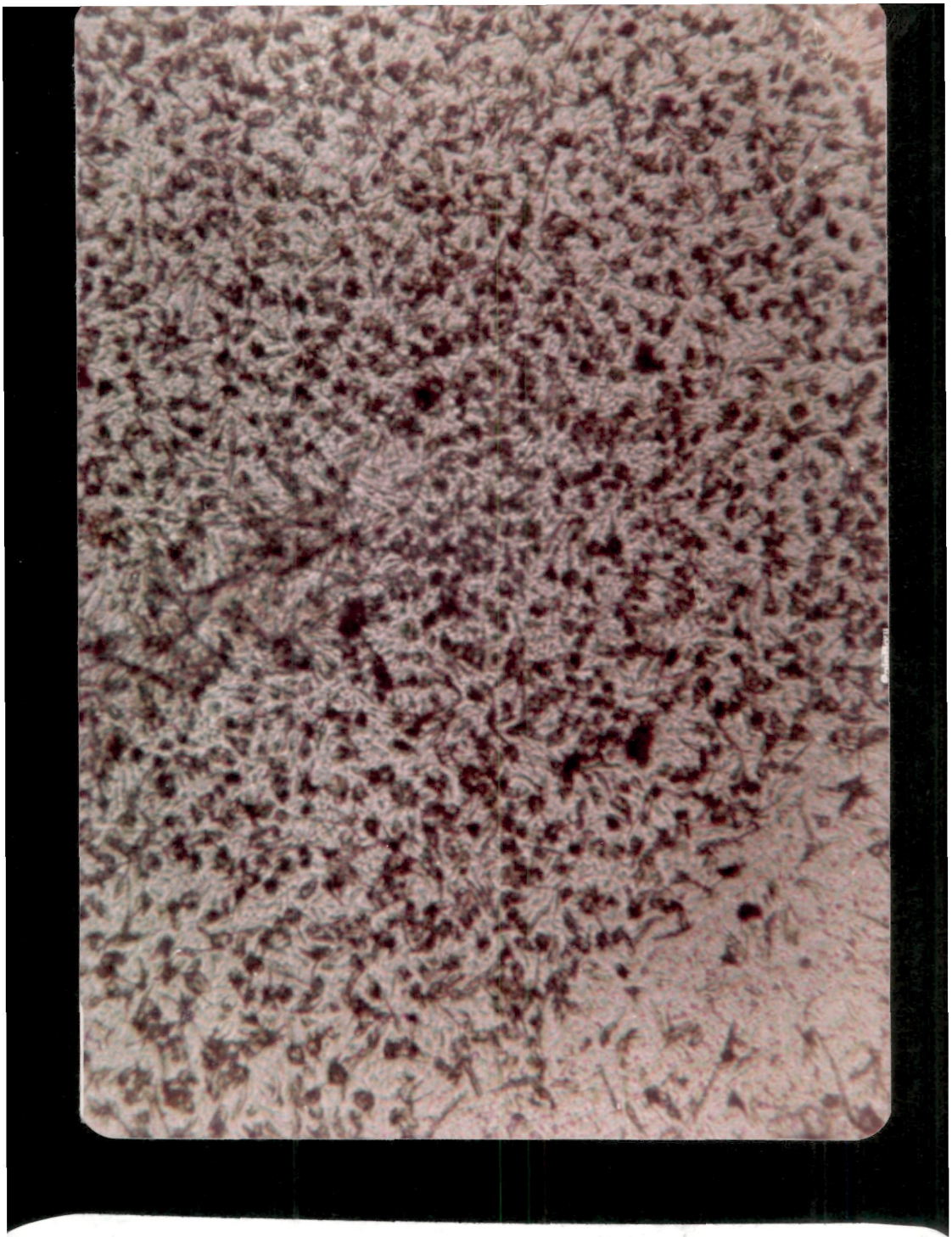

Fig.(5): Cryostat section (20 μm) showing part of Caudoputamen. Note the zinc-zincon reaction in the nucleus (Brown colour) and in the cytoplasm and nerve fibres (Light blue colour). x95

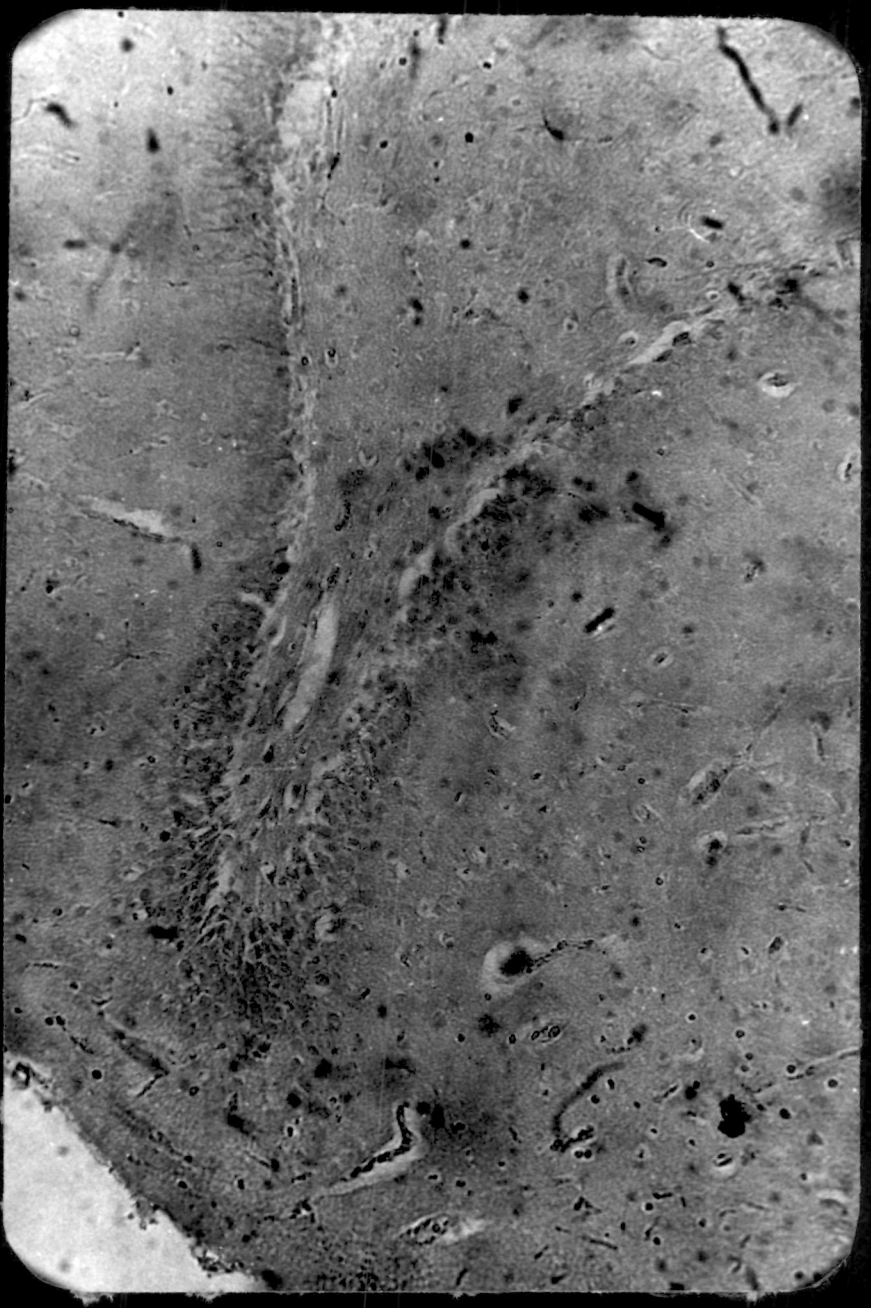

Fig.(6): Paraffine section (9 μm) showing part of hippocampus. Note the sites of Alkaline phosphatase activity stained black in colour. x95

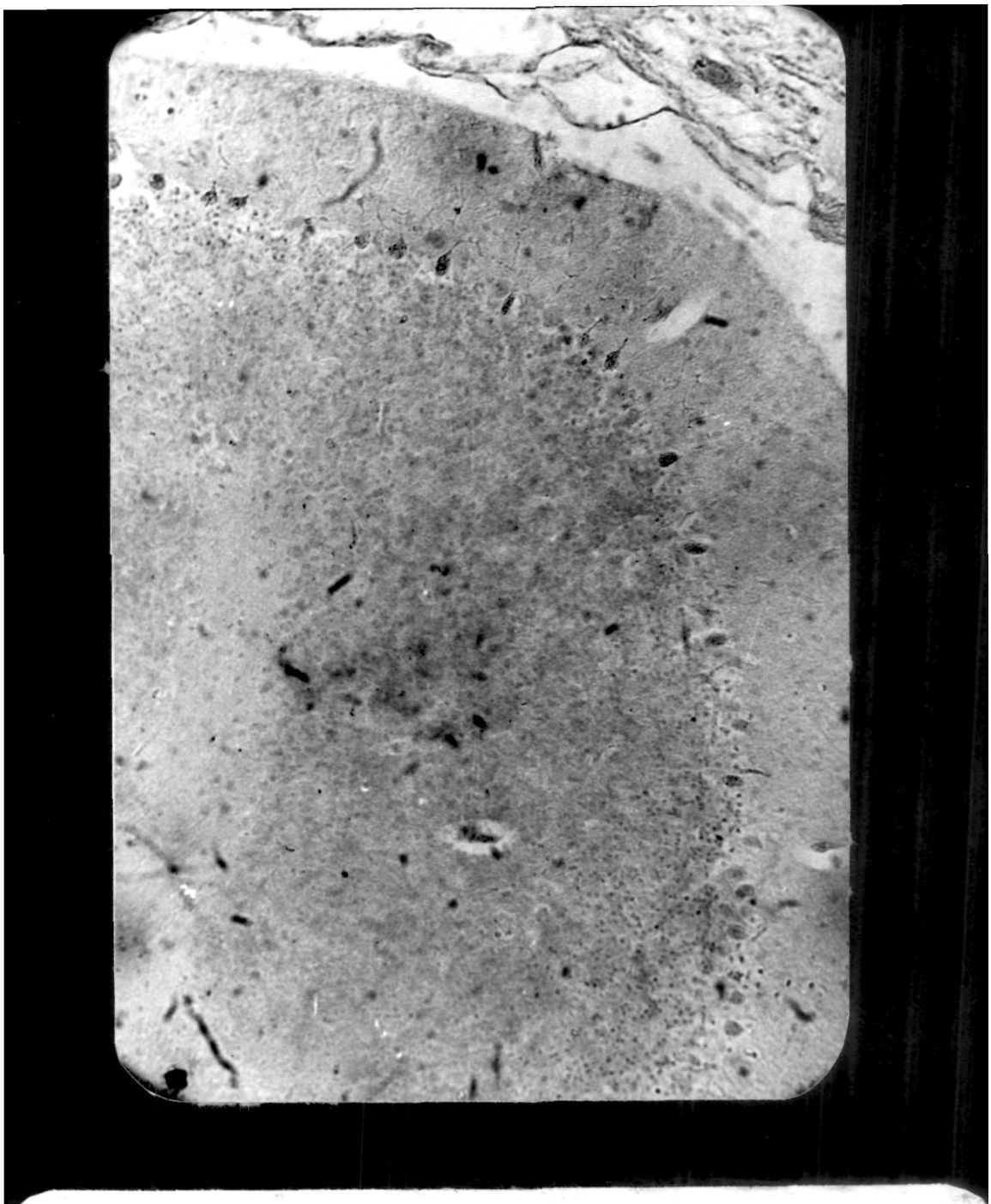

Fig.(7) Paraffin section (9 μm) showing part of cerebellum. Note the sites of alkaline phosphatase activity stained black in colour. x95

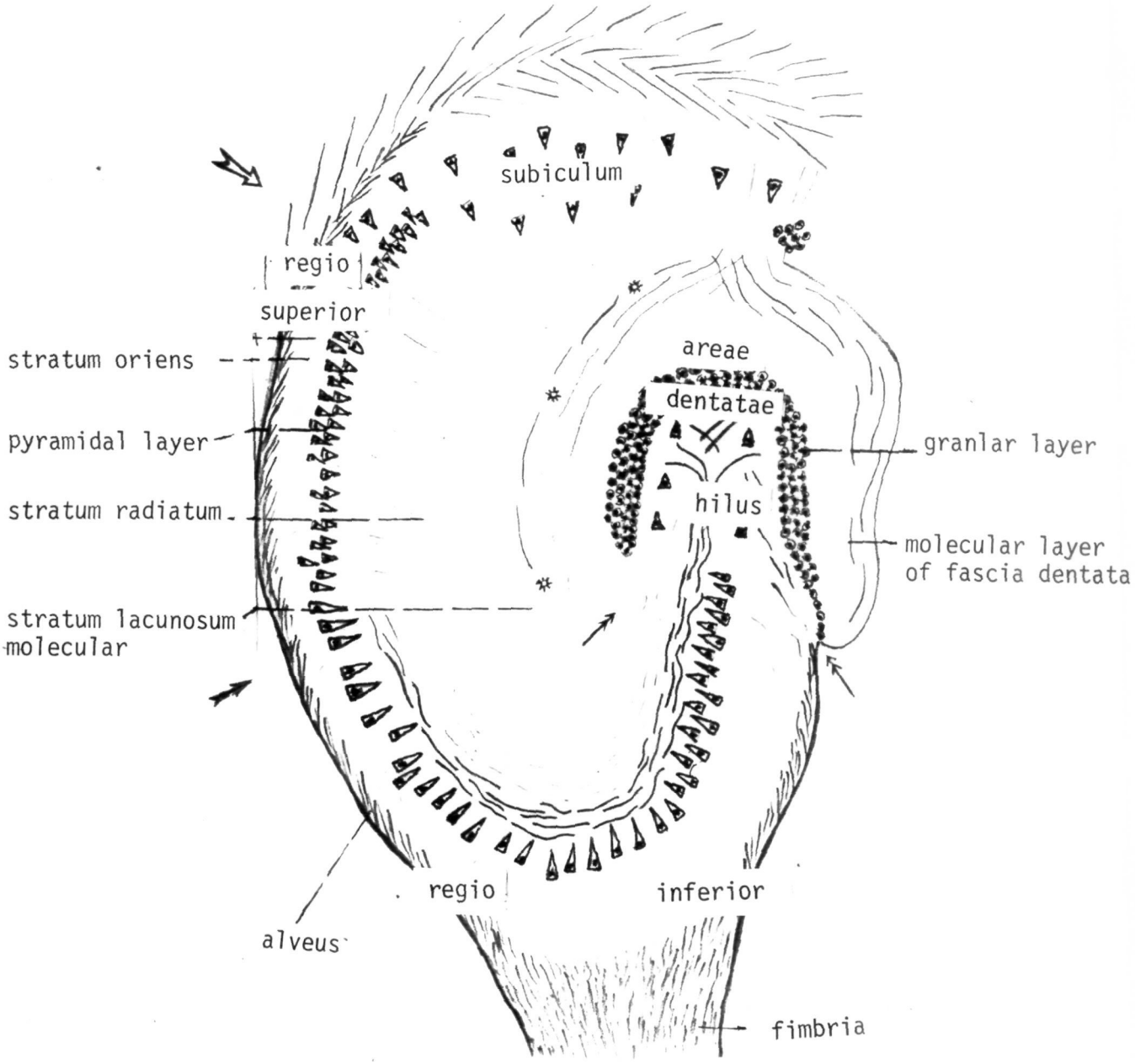

Fig. (8) Schematic drawing of the hippocampal region on a horizontal section at a middle septotemporal level corresponding to the occipital bend. The hippocampus proper is delimited towards the subiculum by open arrow and towards the dentate area by double headed arrows. Its two subfields, the regio superior and the regio inferior, are separated by solid arrow. The obliterated hippocampal fissure is indicated by asterisks.